Development of LC-MS/MS methods for the quantitative determination of hepcidin-25, a key regulator of iron metabolism

D i s s e r t a t i o n

zur Erlangung des akademischen Grades

d o c t o r r e r u m n a t u r a l i u m

(Dr. rer. nat.)

im Fach: Chemie

Spezialisierung: Angewandte Analytik und Umweltanalytik

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät

der Humboldt-Universität zu Berlin

von

M.Sc. Ioana-Monica Abbas

Präsidentin der Humboldt-Universität zu Berlin

Prof. Dr.-Ing. Dr. Sabine Kunst

Dekan der Mathematisch-Naturwissenschaftlichen Fakultät

Prof. Dr. Elmar Kulke

Gutachter/innen:	1. PD Dr. Michael G. Weller
	2. Prof. Dr. Maria Montes-Bayón
	3. Prof. Dr. Michael Linscheid
Tag der mündlichen Prüfung:	12.07.2018

Diese Arbeit ist vom September 2014 bis Mai 2018 unter der Betreuung von PD Dr. Michael G. Weller als kooperatives Projekt an der Bundesanstalt für Materialforschung und -prüfung (BAM), der Universität Oviedo (Spanien) und der Humboldt Universität zu Berlin im Rahmen der Graduiertenschule School of Analytical Sciences Adlershof (SALSA) entstanden.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Michael Weller for his valuable guidance and precious expertise. He has been actively interested in my work and has always been available to advise me. I would also like to thank my second supervisor Prof. Dr. María Montes-Bayón whose support and immense knowledge in the field of mass spectrometry contributed to the completion of this thesis. I am thankful to Prof. Dr. Michael Linscheid for agreeing to review my thesis.

I thank the research group of Protein Analysis at BAM Berlin and Mass Spectrometry & Biomedical Analysis at University of Oviedo for their constant support and encouragement. My sincere thanks to Dr. Andreas Lehmann, Dr. Steffen Weidner, Dr. Franziska Scherz, Dr. Bogdan Bernevic, Dr. Sergio Cueto Díaz and Dr. Mario Corte-Rodríguez for introducing me to analytical techniques that I was unfamiliar with and were necessary for the completion of this work. I would like to acknowledge the excellent teamwork conducted together with Holger Hoffmann and Dr. Ahmed El-Khatib, whom I thank for their technical input and valuable contribution to my research.

I would like to acknowledge Marija Vranic and Prof. Dr. Heiko Möller from the University of Potsdam for fruitful collaboration. I thank Prof. Dr. Dorine Swinkels from Radboud University Nijmegen for providing samples within the round robin exercise.

Thanks to Timm Schwaar, Konrad Löhr and Oleksandr Zavoiura for stimulating discussions and encouragement.

I am grateful to German Initiative of Excellence (DFG), School of Analytical Sciences Adlershof (SALSA) and Bundesanstalt für Materialforschung und -prüfung (BAM) for providing funding for this project.

Thanks to my parents, Marioara and Ioan, and my bother Mihai for supporting me spiritually and assisting me whenever I needed help. Finally, I wish to thank Zaheer for accompanying me throughout this journey with endless support, guidance and wisdom.

Abstract

Isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS/MS) is emerging in the field of clinical chemistry and laboratory medicine as an alternative to immunoassays and is acknowledged as the MS "gold standard" for small biomolecule quantification. Hepcidin-25, a key iron-regulatory peptide hormone discovered in 2000, has revolutionized the understanding of iron disorders and its quantitative determination in biological samples should advance the management of iron related pathology (diagnosis, prognosis and treatment). This study applied LC-MS/MS, using the triple quadrupole (QqQ) mass spectrometer, in a rapid and robust analytical strategy for the quantification of hepcidin-25 in human serum, to be implemented in routine laboratories. For this purpose, two sample preparation strategies and two complementary chromatographic separation conditions were investigated, where the use of acidic mobile phases (0.1% trifluoroacetic acid) was compared with a novel approach involving solvents at high pH (containing 0.1% ammonia). The application of these LC-MS/MS methods to human samples in an intra-laboratory comparison, using the same hepcidin-25 calibrators, yielded a very good correlation of the results. The LC-MS/MS employing trifluoroacetic acid-based mobile phases was selected as a highly sensitive (limit of quantification LOQ of 0.5 µg/L) and precise (coefficient of variation CV<15%) method and was recommended as a reference method candidate for hepcidin-25 quantification in real samples (in the dynamic range of 0.5-40 µg/L). One of the novel aspects of the methodology was the use of amino- and fluoro-silanized autosampler vials to reduce the interaction of the 25-residue peptide to laboratory glassware surfaces. Moreover, this LC-MS/MS method was used for an international round robin study, applying a secondary reference material as a calibrator. By determining the degree of equivalence between the results of the ten participating methodologies, the performance of the method developed in this study was found to be in the optimal range as defined by the International Consortium for Harmonization of Clinical Laboratory Results (ICHCLR). In this work, the formation of hepcidin-25 complexes with copper(II) was investigated. The first reversed-phase chromatographic separation of hepcidin-25/Cu^{2+} and hepcidin-25 (copper "free") was achieved by applying mobile phases containing 0.1% of ammonia (pH 11). LC-MS/MS and high-resolution mass spectrometry (Fourier-transform ion cyclotron resonance (FTICR) MS) were applied for the mass spectrometric characterization of the formed hepcidin-25-Cu(II) species at pH values of 11 and 7.4 respectively. A new species corresponding to hepcidin-25 complexed with two copper ions was identified at high pH.

Zusammenfassung

Die Isotopenverdünnungs-Flüssigkeitschromatographie-Tandem-Massenspektrometrie (ID-LC-MS/MS) entwickelt sich auf dem Gebiet der klinischen Chemie und Laboratoriumsmedizin zu einer Alternative zu Immunoassays und gilt als "Goldstandard" für die Quantifizierung kleiner Biomoleküle. Hepcidin-25, ein 2000 entdecktes Peptidhormon, das eine Schlüsselrolle im Eisenstoffwechsel spielt, hat das Verständnis von Eisenerkrankungen revolutioniert. Es ist zu erwarten, dass dessen quantitative Bestimmung in biologischen Proben die Diagnose, Vorhersage und Behandlung von Eisenstoffwechsel-Krankheiten vorantreibt. In dieser Studie wurde Flüssigchromatographie gekoppelt mit einem Triple-Quadrupol (QqQ) Massenspektrometer (LC MS/MS) in einer schnellen und robusten analytischen Strategie zur Quantifizierung von Hepcidin-25 in menschlichem Serum verwendet, welche letztlich in Routine-Laboratorien genutzt werden soll. Zu diesem Zweck wurden zwei Probenvorbereitungsstrategien und zwei komplementäre chromatographische Trennbedingungen untersucht, wobei eine saure mobile Phase (0,1% Trifluoressigsäure) mit einem neuartigen Ansatz unter der Verwendung einer basischen mobilen Phase (0,1% Ammoniak) verglichen wurde. In einem laborinternen Vergleich beider LC-MS/MS Methoden wurde Hepcidin-25 in humanen Proben unter Verwendung der gleichen Hepcidin-25-Kalibrierstandards quantifiziert und eine sehr gute Korrelation der Ergebnisse ermittelt. Hierbei wurde die Analysestrategie mit saurer,mobiler Phase als hochsensitiv (Bestimmungsgrenze (LOQ) von 0,5 µg/L) und präzise (CV <15%) befunden und als Kandidat einer Referenzmethoden für die Hepcidin-25 Quantifizierung in realen Proben empfohlen (im dynamischen Bereich von 0,5-40 µg/L). Einer der neuartigen Aspekte der Methodik war die Verwendung von Amino- und Fluor-silanisierten Autosampler-Fläschchen, um die Adsorption des 25 Reste umfassenden Peptids an Oberflächen zu reduzieren. Darüber hinaus wurde diese LC-MS/MS-Methode in einer internationalen Ringversuchsstudie eingesetzt, bei der ein sekundäres Referenzmaterial als Kalibrierstandard verwendet wurde. In dieser Ringversuchsstudie wurde gemäß des International Consortium for Harmonization of Clinical Laboratory Results (ICHCLR) die hier präsentierte Methode im Vergleich mit neun anderen Methoden als optimal bewertet. In dieser Arbeit wurde die Bildung von Hepcidin-Komplexen mit Kupfer(II) untersucht. Die erste Umkehrphasen-chromatographische Trennung von Hepcidin-25/Cu(II) und Hepcidin-25 (Kupfer "frei") wurde unter Verwendung von mobilen Phasen mit 0,1% Ammoniak (pH 11) erreicht. LC-MS/MS und hochauflösende Massenspektrometrie (Fourier-Transformations-Ionenzyklotronresonanz (FTICR) MS) wurden für die massenspektrometrische Charakterisierung der gebildeten Hepcidin-25-Cu(II)-Spezies bei pH-Werten von 11 bzw. 7,4 verwendet. Eine neue Spezies, Hepcidin-25 komplexiert mit zwei Kupferionen, wurde bei hohem pH identifiziert.

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Abbreviations

ACD	Anemia of chronic disease
ACN	Acetonitrile
AI	Anemia of inflammation
APCI	Atmospheric pressure chemical ionization
ATCUN	Amino terminal Cu(II)- and Ni(II)-binding (site)
CHCA	α-Cyano-4-hydroxycinnamic acid
CID	Collision-induced dissociation
CKD	Chronic kidney disease
CV	Coefficient of variation
DAD	Diode-array detector
DCYTB	Duodenal cytochrome B
DMT1	Divalent metal transport protein
EDTA	Ethylenediaminetetraacetic acid
EI	Electron impact ionization
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
ESA	Erythropoietin stimulating agent
ESI	Electrospray ionization
FA	Formic acid
Fe-Tf	Transferrin-bound iron
FPN	Ferroportin
FTICR	Fourier-transform ion cyclotron resonance
FWHM	Full Width of the peak at Half its Maximum height
HAMP	Hepcidin antimicrobial peptide
Hb	Hemoglobin
HCP1	Heme carrier protein 1
Hep-25	Hepcidin-25
HFE	Human hemochromatosis protein
HHS	Hepcidin Harmonization Study
HMJ	Haemojuvelin
HPLC	High-performance liquid chromatography
HR-MS	High-resolution mass spectrometry
HSA	Human serum albumin
ID	Isotope dilution
IDA	Iron deficiency anemia
ID-LC-MS/MS	Isotope-dilution liquid chromatography - tandem mass spectrometry
IL-6	Interleukin 6
IRE	Iron-responsive elements
IRIDA	Iron-refractory iron deficiency anemia
IRP	Iron-regulatory proteins

IS	Internal standard
LA	Low adsorption
LC-MS	Liquid chromatography - mass spectrometry
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
LEAP-1	Liver-expressed antimicrobial peptide
MALDI	Matrix-assisted laser desorption/ionization
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MT-2	Matriptase-2
NGAL	Neutrophil gelatinase-associated lipocalin
NMR	Nuclear magnetic resonance
NTBI	Non-transferrin-bound iron
PBS	Phosphate-buffered saline
PDB	Protein Data Bank
PP	Polypropylene
RIA	Radioimmunoassay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high-performance liquid chromatography
RT-PCR	Real-time quantitative polymerase chain reaction
SRM	Selected reaction monitoring
Tf	Transferrin
TFA	Trifluoracetic acid
TFR	Transferrin receptor
TOF	Time-of-flight
USF2	Upstream stimulatory factor 2
UV-Vis	Ultraviolet-visible (spectroscopy)
WHO	World Health Organization

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Introduction

The discipline of clinical chemistry, earlier known as chemical pathology, arose from the attempts to apply chemistry into medicine [1]. A clinical chemist perceives the patients as "black boxes", complex metabolic machines that utilize molecules to generate energy and to defy entropy. Clinical diagnosis, at its very core, represents important data collection from this "box", separation of signal from noise and eventually assigning an interpretation to this signal. This implies that the information acquired from a body fluid sample can be processed to deduce knowledge about the state of the individual that it came from [2].

With the publication of the first comprehensive textbook in this field by R. J. Henry in 1964, the ground stone for the application of analytical chemistry in the framework of laboratory medicine was set. Together with other relevant concepts in the field, such as accuracy, precision, control charts, significant figures, and normal values, this book covered one of the first breakthroughs in clinical pathology represented by photometry. In addition, analytical methods were described for various proteins and peptides found in biological fluids, inorganic ions, blood pH and gases, electrolytes, enzymes, carbohydrates and metabolites, vitamins, lipids and others [3]. This was followed by the successful introduction in clinical chemistry of antibody-based ligand binding assay ("immunoassay") in the 1960s and of the gas chromatography-mass spectrometry in the 1970s. However, it was the introduction of non-disintegrating soft ionization techniques as atmospheric pressure ionization in the 1990s that ultimately made most of the biologically important analytes (endogenous metabolites as well as xenobiotics) amenable to highly specific mass spectrometric analyses [4]. Nowadays, liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) is accepted as the "gold standard" for quantification of many biomolecules in clinical chemistry and laboratory medicine [5].

Going back to the metaphoric context of the "black box", the disorders of iron metabolism exhibit significant contribution to its unresolved puzzles. According to the World Health Organization (WHO), iron deficiency affects more people than any other condition, constituting a public health condition of epidemic proportions [6]. The diagnosis of iron-related pathology assumes the evaluation of the players involved in iron homeostasis. By such, the present study intends to focus on the clinical assessment of the primary iron regulator using LC-ESI-MS/MS. By putting it in a very concise manner, one could say that this research work is devoted to find a connection between life and the test tube.

1. Theoretical background

1.1. Iron

Iron (Fe) is the most frequent element on Earth after oxygen and silicon, contributing to the composition of all its three main layers: crust, mantle and core [7, 8]. Due to its high reactivity, iron is rarely found in its elemental state. In this respect, the divalent, ferrous iron and the trivalent, ferric iron are the oxidation states of highest importance. However, iron appears in a variety of other states, for example the hexavalent ferrate [9].
This element presents a vast diversity of functions in the biosphere. All organisms with rare exceptions among bacteria need iron from the environment to meet their metabolic needs [10]. In humans, iron is an essential nutrient used by iron-containing proteins for oxygen transport and storage or as a catalyst for redox reactions [11].
In order to establish a good understanding of the factors involved in maintenance of iron balance and the mechanisms resulting in pathological states, iron metabolism and its regulation in the human body will be discussed in detail in the following section.

1.1.1. Human iron metabolism

When addressing iron metabolism in humans, two levels of regulation are to be identified. The lower level is cellular and refers to the mechanisms of iron uptake, transport and intracellular use. The upper level or the systemic level comprises the correlation between iron uptake and iron sensing, iron demand signaling and signal transfer to the iron stores [10]. A multitude of regulators, transporters and enzymes are involved in the management of iron balance at both the cellular and systemic levels. The interplay of these actors in iron absorption, distribution, storage and recycling will be addressed further.

1.1.1.1. Intestinal iron absorption

Generally, humans procure iron exclusively from the diet. Typically, dietary iron is categorized as heme and non-heme iron. Heme iron is mostly found in meat as part of hemoglobin or myoglobin, while non-heme iron consists of various forms of iron of both vegetal and animal origin (meat, fruits, vegetables, grains, nuts etc.) [12, 13]. The absorption of iron takes place in the duodenum, where new iron is imported in the body by epithelial cells called enterocytes [14]. In this regard, Figure 1.1 presents the biochemical pathway of iron uptake by enterocytes [15], wherein non-heme iron is transported inside the intestinal cell by a divalent metal transport protein (DMT1) [16]. Most of the dietary non-heme iron occurs to be in the +3 oxidation state and, prior its uptake, requires reduction to the more soluble ferrous state by the duodenal cytochrome B (DCYTB), also known as ferrireductase [17].

Further, the absorption of heme iron is not fully elucidated. However, it is suggested that heme is taken in by receptor-mediated endocytosis through the activity of heme carrier protein 1 (HCP1). Heme is likely further degraded by heme oxygenase, and the released iron enters the same storage and transport pathways as non-heme inorganic iron [12, 18].

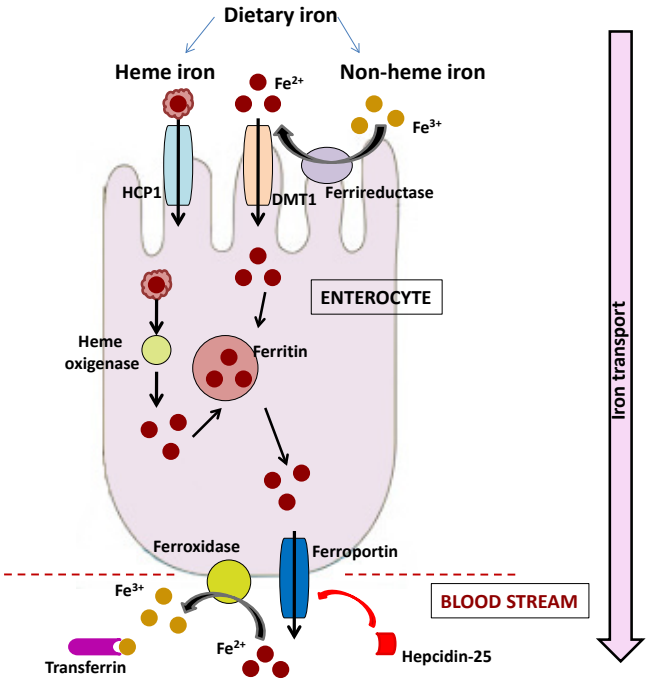

Figure 1.1: Iron absorption in the enterocyte [15].

Once iron penetrates the duodenal cell, the ferrous iron may follow two paths. The amount that is not needed for immediate use is stored in ferritin (up to 4500 atoms of iron/ferritin molecule [19]), an ubiquitous protein within the enterocyte [20, 21]. This is however lost after two-three days, owing to the sloughing of enterocytes into the gut lumen. [22]. Alternatively, depending on the body needs, iron can be released in the blood stream [14] via ferroportin (FPN) [23]. This export of cellular iron demands an associated ferroxidase activity. In this vein, ceruloplasmin, a circulating multicopper oxidase, and hephaestin, a homolog of ceruloplasmin, seem to be responsible for oxidation of Fe^{2+} to Fe^{3+} in the duodenum [22].
It should be noted that FPN, also known as SLC40A1, is the sole known iron exporter [11] and serves as a target protein for iron regulation via hepcidin-25 (Hep-25). This will be addressed in detail in *section 1.2*.

1.1.1.2. Iron distribution

Once released in the blood, absorbed iron is almost exclusively bound to transferrin (Tf), an abundant, high affinity iron-binding protein [22]. Tf presents two binding sites for iron, therefore three different forms, namely apo-transferrin (not bound to iron), monoferric-transferrin and diferric-transferrin, can be identified in plasma [24]. In physiological conditions, up to 35% saturation of transferrin is achieved. In iron-overload though, iron levels can exceed the binding

capacities of plasma Tf and consequently, a supplementary iron compartment, non-transferrin-bound iron (NTBI), occurs in plasma [25, 26]. Tf maintains iron in its soluble form and prevents the ferric iron from precipitating out of aqueous plasma [14]. Transferrin-bound iron, Fe-Tf, is further distributed to sites of use and storage in the human body.

In this regard, two thirds of the body iron is transported to the erythrocytes in the bone marrow, where most of it is used for the production of erythroid precursors and red blood cells [14]. The uptake of Fe-Tf by erythroid precursors takes place by receptor-mediated endocytosis and is activated by cell surface transferrin receptors (TFRs). The disruption of Tf-TFR cycle can lead to shortfalls in the production of erythrocytes [27]. It should be mentioned that most of the other cells acquire iron by similar means, namely through TFR expressed on their membrane surface. Further tissues that need considerable amounts of iron are the muscles. Iron participates in the production of myoglobin, a protein seemingly responsible for oxygen delivery in active muscle [28]. Iron also proved to be a vital growth factor in the early development of kidneys. Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin (NGAL), is involved in innate immunity at renal level by sequestrating iron containing siderophores that sequentially limit bacterial growth [29]. Finally, iron is present within cells incorporated in various hemeproteins such as cytochromes, peroxidases or catalases, to facilitate their biological functions.

1.1.1.3. Iron storage and recycling

Two types of cells are relevant for iron storage: macrophages and hepatocytes. As mentioned before, the iron stored by ferritin within the enterocyte leaves the body when the intestinal cells die, thus enterocytes are not considered as member of the iron storage system [14].

Macrophages are white blood cells found essentially in all tissues, that ingest foreign substances and microbes in a process known as phagocytosis [30]. In the context of iron metabolism, the macrophages, mainly from the spleen, are responsible for recycling iron from senescent erythrocytes. In this process, old and damaged erythrocytes are recognized by macrophages, and phagocytosed. Their lysis results in the liberation of iron from hemoglobin through catabolism by heme oxygenase [14, 31]. Iron can either be stored further in the macrophages, or released in plasma via ferroportin [32]. Similar to iron export from enterocytes, ferroportin is critical for the release of iron from macrophages, and can be regulated to modify the amount of iron stored/released by way of hepcidin-25 (see *section 1.2.*) [22].

Hepatocytes or liver cells serve as the major depot for the storage of surplus iron [14]. These can accommodate both Fe-Tf and NTBI. For this purpose, the uptake of iron by the hepatocytes is mediated mainly by TFR. Once inside the liver cells, most of the iron is stored in the form of ferritin, which, based on body requirements, can be mobilized to the blood stream through the activity of ferroportin [22].

1.1.2. Systemic iron homeostasis

1.1.2.1. Circulation of iron in the human body

Total body iron in average human adults is known to be 2-4 g. In this context, the circulation of iron in humans is described in Figure 1.2. Only 1-2 mg of iron needs to be absorbed daily from the diet by the enterocytes to compensate for iron losses [33]. Iron absorption must be thoroughly regulated to keep the iron balance, since there is no mechanism for the excretion of surplus iron [34, 33]. For this reason, iron losses occur essentially only through desquamation of epithelial cells in the intestine and skin, minor hemorrhaging or menstruation (in women) [35].

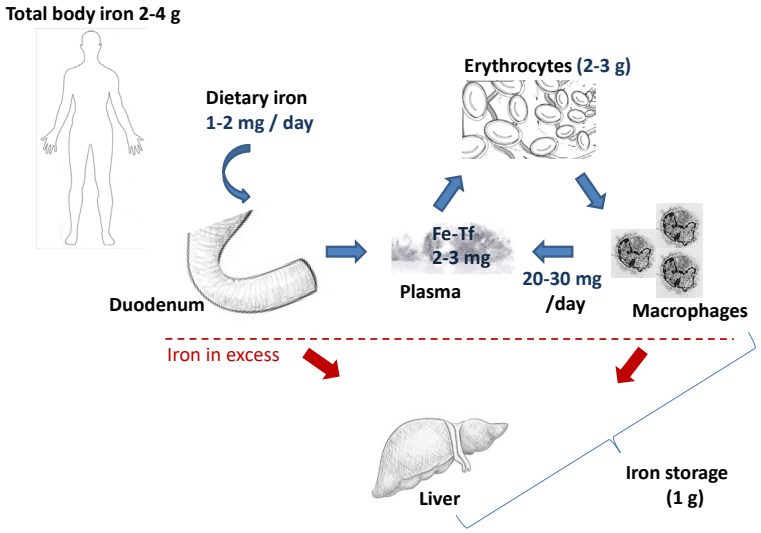

Figure 1.2: Iron distribution in the human body [36].

The absorbed iron is transported in plasma via transferrin to the bone marrow and developing tissues. The largest fraction of iron in the body is contained in the hemoglobin of the red blood cells. Erythrocytes encompass 2-3 g of iron, while plasma contains only 2-3 mg of iron bound to transferrin (Fe-Tf). The recycled iron from senescent erythrocytes amounts to be in the range of 20-30 mg/day. When the iron-binding capacity of transferrin is achieved, iron is deposited in parenchymal tissues. About 1 g of iron is stored in the cytoplasmic ferritin of the hepatocytes and the macrophages in the spleen [35, 36].

1.1.2.2. Iron regulation

The lack of an iron-excretion system implies that the iron homeostasis is maintained by a good orchestration of iron absorption, distribution and storage.

At cellular level, iron is regulated by two iron-regulatory proteins (IRP), which, when iron levels are low, bind to RNA stem-loop iron-responsive elements (IRE) [22]. IRP1 and IRP2 are part of the family of iron-sulfur cluster isomerase [37] and are identified in the mRNA (messenger ribonucleic acid) encoding proteins involved in iron transport and storage. IRP1 binds to IREs in the mRNA encoding ferritin and blocks the translation of the iron storage protein.

IRP2 follows a different mechanism by binding to the IREs found in the TFR mRNA, allowing the increase of TFR production in iron deficit [22, 38].

At systemic level, the organs majorly implicated in the handling of iron as presented in *section 1.1.1.* include the duodenum involved in dietary iron absorption, the liver which is the main depot of iron storage, and the spleen containing macrophages that recycle iron from old erythrocytes. Thus, three different cell types are engaged in iron metabolism, namely enterocytes, hepatocytes and macrophages. These present different pathways for iron uptake, but a single pathway for iron export into the blood stream represented by ferroportin [11, 33].

It has been postulated for a long time that a circulating factor operates between these organs to synchronize iron metabolism [25]. In recent past, this role was attributed to a newly discovered peptide hormone called hepcidin-25 [39], which binds to ferroportin, causing its internalization and degradation within lysosomes [40] (Figure 1.3). Ever since, hepcidin-25 became the main player in iron homeostasis (see *section 1.2.4.*).

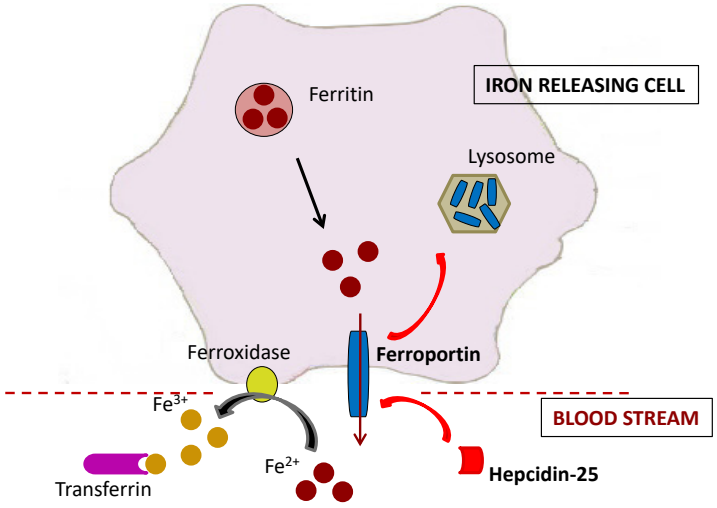

Figure 1.3: The regulation of iron by hepcidin-25. Hepcidin-25 binds ferroportin found in iron releasing cells (enterocytes, hepatocytes and macrophages) and causes its internalization and degradation within the lysosome.

Prior the uncovering of hepcidin-25, the stimuli known for modulation of iron homeostasis were erythropoiesis, hypoxia, iron deficiency or overload and inflammation. The same stimuli were found to regulate the expression of hepcidin-25 [22], which will be discussed in detail in the *section 1.2.3.*

1.1.3. Disorders of iron metabolism

The iron regulator hepcidin-25 is involved in the pathogenesis of several iron-related disorders with a high public health impact worldwide. Details on these disorders, their cause and link to hepcidin-25 expression will be briefly discussed further.

1.1.3.1. Iron deficiency

Iron deficiency anemia (IDA)

Anemia is a common condition with heterogeneous etiologies , with a worldwide prevalence of around 33% of the population (2010) [41]. Anemia generally refers to the decrease in the red blood cell levels or hemoglobin (Hb) levels caused by an imbalanced relationship between erythropoietin, iron, erythropoiesis and hepcidin-25 [42, 43]. Erythropoietin represents a glycoprotein hormone secreted by the kidney that stimulates red blood cell production (erythropoiesis) [44, 45].

Iron deficiency (ID) is the most common cause for anemia, affecting especially children, women and the elderly. ID syndromes include two main forms: absolute and relative. Absolute ID occurs when total body iron stores are low or depleted, while relative or functional ID arises when total body iron stores are normal or even increased, but stimulated/increased erythropoiesis induces iron demand that exceeds the supply [41]. Taking this into account, absolute iron deficiency is known as iron deficiency anemia (IDA), while relative iron deficiency is usually associated to inflammatory diseases, hemoglobinopathies, chronic hemolytic anemias or to the treatment with erythropoiesis stimulating agents [42, 46].

According to WHO (World Health Organization), IDA is the most widespread nutritional disorder in humans [6], with more than one billion people affected worldwide. The main cause of IDA in children and pregnant women is the high iron demand, while gastrointestinal bleeding (due to high drug intake) and malabsorption are the most common causes of IDA in older individuals [46]. Moreover, secondary to blood losses or insufficient dietary intake, low hepcidin-25 levels have also been associated with iron deficiency [47] (see *section 1.2.4.*).

A distinct type of iron deficiency anemia is iron-refractory iron deficiency anemia (IRIDA), characterized by low levels of iron that cannot be corrected by oral iron therapy. IRIDA is a genetic disease caused by mutations in TMPRSS6, which encodes the membrane protease matriptase-2 (MT-2). This acts as inhibitor for the hepatic production of hepcidin-25. Hence, the lack of MT-2 generates elevated levels of hepcidin-25 which lead to hypoferremia by blocking iron outflow to the blood stream [48] (see *section 1.2.4.*).

Anemia of chronic disease

Anemia of chronic disease (ACD) or anemia of inflammation (AI) is a condition that can be associated with various chronic inflammatory disorders such as inflammatory bowel disease, cancer, chronic infections or rheumatological disorders. ACD can be defined as a moderate anemia, characterized by persistent and high production of pro-inflammatory cytokines [49, 50].

In this regard, IL-6 (interleukin 6) and other pro-inflammatory cytokines induce hepcidin-25 production (see *section 1.2.4.2.*), resulting in the blockage of iron efflux. Therefore, iron is sequestrated in the macrophages, resulting in low serum iron (Fe-Tf) levels which cause further inadequate erythropoiesis [47, 51]. Other mechanisms for decreased erythropoiesis or anemia

associated with ACD are shortened erythrocyte lifespan due to macrophage activation or erythropoietic suppression due to direct effect of cytokines on erythroid precursors [35].
ACD presents hypoferremia and mild anemia that can be treated by oral iron administration, blood transfusions or erythropoietic agents. However, disagreements exist with regard to the benefits of iron therapy, since duodenal iron absorption will be down-regulated by the increased levels of hepcidin-25 [51] (see *section 1.2.4.2.*).

Anemia associated with chronic kidney disease

Chronic kidney disease (CKD) is a general term used for heterogeneous disorders affecting the structure and function of the kidney [52]. With a global prevalence of 8% , CKD is a worldwide public health problem [53] which is generally associated with old age, diabetes, hypertension, obesity, cardiovascular disease and anemia [52]. This anemia shares the features of anemia of chronic disease, with additional typical characteristics that contribute to decreased erythropoiesis, such as renal insufficiency and accumulation of uremic toxins [51].

Table 1.1: Classification of CKD [54, 53].

CKD Stage	**Classification by severity** GFR (mL/min/1.73 m^2)	**Classification by treatment**
1 (normal or ↑ GFR)	≥ 90	Kidney transplant
2 (mild ↑ GFR)	60-89	Kidney transplant
3 (moderate ↑ GFR)	30-59	Kidney transplant
4 (severe ↑ GFR)	15-29	Kidney transplant
5 (end stage renal disease ESRD)	<15	Dialysis

CKD represents a severe chronical condition classified based on the level of glomerular filtration rate (GFR), a calculated parameter which characterizes the renal excretion function [53] (Table 1.1). In this sense, CKD has no etiotropic treatment and eventually gets to a point where the kidneys stop working and the only treatments options are either the kidney transplant or dialysis. Hence, symptomatic treatment is applied to improve the quality of life of the patients. Generally, ESAs (erythropoietin stimulating agents) are used to correct the anemia due to inadequate erythropoietin production, historically attributed as the main cause for anemia. However, 10-20% of the cases are not responsive to this therapy [53]. This is because CKD patients also suffer from an iron-restrictive disorder due to elevated levels of hepcidin-25 which contributes to decreased erythropoiesis [35]. Several studies found significantly high levels of circulating Hep-25 in patients suffering from CKD, especially ESRD patients on dialysis [55]. This can be explained by the up-regulation of the peptide in uremia and other inflammatory states (see *section 1.2.3.2.*) and by a low renal clearance of hepcidin-25 due to impaired renal function [56]. In view of this, Hep-25 modulation could represent an alternative treatment to ESAs therapy by limiting the hypoferremia.

1.1.3.2. Iron overload

Hereditary Hemochromatosis

Hemochromatosis is a general term used since the 17th century for iron overload and accumulation in the body [57].

Hereditary hemochromatosis (HH) is a group of genetic iron disorders caused mainly by mutations of human hemochromatosis protein (HFE), discovered in 1997 as the HH responsible gene [58]. HH type 1, also known as classic HH or HH HFE-related, is the most common type of hemochromatosis [59], affecting mostly Caucasians [60]. HH type 2 or juvenile hemochromatosis is a rare syndrome caused by HJV mutations (90% of the cases) and by HAMP mutations [61]. Hemochromatosis type 3 prevails rarely as well [62], and, together with HH type 2, is affecting all races worldwide [63]. HH type 4, also called ferroportin disease, is a rare autosomal dominant disorder, unlike the other three types, which are autosomal recessive diseases. It is characterized by a distinct mechanism of loss-of-function mutation of ferroportin [64]. Other hereditary iron overload disorders are aceruloplasminemia, atransferrinemia or neonatal hemochromatosis [59].

Table 1.2:The genetic basis of hereditary hemochromatosis (HH) [65].

HH Type	Mutated gene	Encoded protein
1	HFE	HFE (Human hemochromatosis protein)
2A	HJV or HFE2	HJV (Haemojuvelin)
2B	HAMP or HFE2B	Hepcidin-25
3	TRF-2 or HFE3	TFR 2 (Transferrin receptor 2)
4	SLC40A1	FPN (Ferroportin)

Generally, HH types 1-3 are characterized by an excess of transferrin-bound iron (Fe-Tf). In this context, higher levels of circulating iron are caused on one hand by hyperabsorption of dietary iron with subsequently elevated iron export from the enterocytes, and by increased release of iron from the macrophages on the other hand [66, 63]. On this matter, the abnormal behavior of macrophages and enterocytes is linked to the unrestricted and ferroportin-mediated iron export induced by hepcidin-25 deficiency [67, 68, 63]. Levels of this peptide are low either because of mutations of HFE, HJV and TfR genes, which regulate hepcidin expression (see *section 1.2.4.2.*), or due to direct mutation of HAMP gene (HH type 2B) which encodes hepcidin-25 (Table 1.2).

Iron accumulation, if not treated, particularly in parenchymal cells, results in damage of intracellular structures by catalysis of the production of reactive oxygen species (ROS) [69, 63]. As a result, organs such as liver, pancreas, heart and gonads are impaired [63, 70]. In this respect, hemochromatosis is currently managed by bloodletting or phlebotomy to clear the iron excess. Although this treatment is effective, it is not suitable to all patients due to poor patient compliance caused by precarious vascular access. In light of this, therapeutic modulation of hepcidin-25 may be a future alternative treatment for HH [71, 35].

Iron-loading anemias

The term "iron-loading anemia" indicates anemias characterized by defective erythropoiesis and iron overload [72]. In this sense, the most common example is β-thalassemia, an autosomal recessive disease of hemoglobin (Hb). Patients suffering from this disorder present mutation of the gene encoding the β-globin chains of the Hb tetramer (which comprises of two alpha globin and two beta globin chains) which causes the apoptosis of erythroid precursors in the process of erythropoiesis [73, 35]. The resulting anemia triggers erythropoietin production. This generates further the production of erythroid precursors, which will not correct the anemia because these precursors will undergo apoptosis [35]. Further, iron overload is generated by a hyperabsorption of iron, caused by low levels of hepcidin-25, as in the case of hereditary hemochromatosis [74].

Considering this, regular blood transfusions are acknowledged as the only way to sustain life in ß-thalassemia patients. Moreover, after each 12-14 transfusions, chelation therapy is required, which is administered 5–7 days a week by 12-hour continuous subcutaneous infusion via a portable pump [73]. In this regard, similarly as in the case of HH, hepcidin-25 modulation could be used as an alternative therapy in the future, which might help to resolve the precarious patient compliance [75].

1.2. Hepcidin

The discovery of the peptide hormone hepcidin-25 and its crucial function in iron homeostasis led to a significant upgrade in the understanding of the systemic iron level [10]. Hepcidin-25 is considered nowadays the main iron regulator [35, 76]. Thus, over the past two decades, elucidation of hepcidin activity, its structure and levels in biological fluids, have been the center of attention for several research groups worldwide. A comprehensive overview of the achievements in hepcidin research will be presented further.

1.2.1. The discovery of hepcidin

Hepcidin was discovered concurrently by two independent groups almost twenty years ago.Krause *et al.* isolated hepcidin in 2000 from human blood ultrafiltrate and characterized it as a cysteine-rich peptide with antimicrobial activity, termed LEAP-1 (liver-expressed antimicrobial peptide). LEAP-1 was identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) with a molecular mass of 2789.8 Da. The cysteine content determination using carboxamidomethylation revealed eight cysteine residues. Further sequence analysis performed by Edman degradation completed the primary structure of LEAP-1 as follows: DTHFPICIFCCGCCHRSKCGMCCKT. Expression of the LEAP-1 gene examined by real-time quantitative RT-PCR (reverse transcription polymerase chain reaction) was mainly detected in the liver, and to a much lower extent, in the heart and brain. Additionally,

radial diffusion assays revealed several bacteria including *Bacillus megaterium*, *Bacillus subtilis*, *Micrococcus luteus*, *Staphylococcus carnosus* and *Neisseria cinereal* exhibiting susceptibility to LEAP-1 treatment [77].

Around the same time, Park *et al.* isolated the same cysteine-rich peptide hormone from human urine and, mainly because of its hepatic origin and antimicrobial activity, named it hepcidin. The authors determined that the human hepcidin gene (HAMP, OMIM 606464) was located on chromosome 19 and encoded an 84-residue precursor protein. This so-called pre-prohepcidin contained a 24-amino acid signal peptide at the N-terminus which was enzymatically cleaved to produce a 60-amino acid prohepcidin. Then, prohepcidin was further processed by proteolysis to generate the mature hepcidin. The average mass of the peptide analyzed by MALDI-TOF MS was 2789.40 Da. The 25-amino acid sequence determined by Edman degradation was identical to the one reported by Krause *et al.*. Mass spectrometric (MS) data confirmed that all 8 cysteines were engaged in intramolecular disulfide bonds, with no assignment of the pairings determined at this point. Additionally, two N-truncated variances of the 25-residue peptide, named hepcidin-20 and -22 respectively, were identified. Further MS measurements determined that most of the hepcidin found in urine is hepcidin-20 (Hep-20) and hepcidin-25 (Hep-25). Hepcidin-22 (Hep-22), however, only occured in minute amount in urine. Hep-20 and Hep-25 were also tested for antimicrobial activity using the colony-forming unit assay (CFU). Both peptides presented antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and group B *Streptococcus*, and antifungal activity against *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger* [39]. However, the half maximal inhibitory concentration (IC_{50}) determined for the antimicrobial activity of hepcidin-25 was 14-18 µM, whereas the mean physiological level of the peptide is around 3.5 nM (10 µg/L) (see *section 1.3.3.*).

In 2001, Pigeon *et al.* determined overexpressed hepcidin mRNA in the liver of iron-overloaded mice by Northern blot analysis [78]. Additionally, Nicolas *et al.* performed PCR experiments that showed severe iron deficiency anemia in transgenic mice overexpressing liver hepcidin [79]. These findings suggested that hepcidin exhibits an additional role distinct from its antimicrobial activity, namely it is involved in iron regulation (see *section 1.2.4.*).

Hepcidin was further identified in numerous species [80-82], with a total of more than 250 entries of hepcidin sequences on protein sequence database UniProt by 2017 [83].

1.2.2. Structure of hepcidin

1.2.2.1. NMR structure of hepcidin-25

In 2002, Hunter *et al.* investigated the structure of synthetic human Hep-25 and Hep-20 using nuclear magnetic resonance (NMR) spectroscopy. Results of these examinations showed the existence of an amphipathic structure, which consists of a distorted β-sheet with a hairpin loop, stabilized by four intramolecular disulfide bonds. Using structure calculations and dynamic signatures in the NMR spectra, the S-S connectivity was attributed between Cys7-Cys23 (1-8),

Cys10-Cys22 (2-7), Cys11-Cys19 (3-6) and Cys13-Cys14 (4-5), with the latter S-S bond corresponding to an unusual adjacent bond. Also, the authors reported that Hep-25 tends to aggregate, in contrast to Hep-20. These aggregation properties were connected to the hydrophobic residues of the NH_2-terminus, namely Phe4, Phe9 and Pro5 [84]. However, because the resonances from the two vicinal cysteines Cys13 and Cys14 could not be detected due to exchange broadening, the NMR data presented was not complete.

Figure 1.4: Amino acid sequence of hepcidin-25 with S-S connectivity.

In 2009, Jordan *et al.* suggested a different S-S pairing for human hepcidin-25 between Cys7-Cys23 (1-8), Cys10-Cys13 (2-4), Cys11-Cys19 (3-6) and Cys14-Cys22 (5-7) (Figure 1.4). Disulfide mapping was achieved by chemical analysis employing partial reductive alkylation in addition to NMR spectroscopy. Moreover, previously encountered difficulties in the NMR analysis were overcome by performing experiments at different temperatures (325 K and 253 K). This permitted the evaluation of exocyclic conformations for the critical cysteines (Cys13 and Cys14), with the establishment of the solution NMR structure of hepcidin-25 with the currently accepted S-S pairing (PDB code 2KEF) (Figure 1.5). The S-S connectivity was confirmed for native human hepcidin-25 (extracted and purified from urine), synthetic hepcidin-25 (synthetized and folded "in house") and two types of recombinant hepcidin-25 (expressed in Chinese hamster ovary (CHO) cells and *Escherichia coli*).

However, the authors reported that the N-terminus was highly flexible and the electron density of the first five residues was insufficient to accurately assign the position of the residues [85].

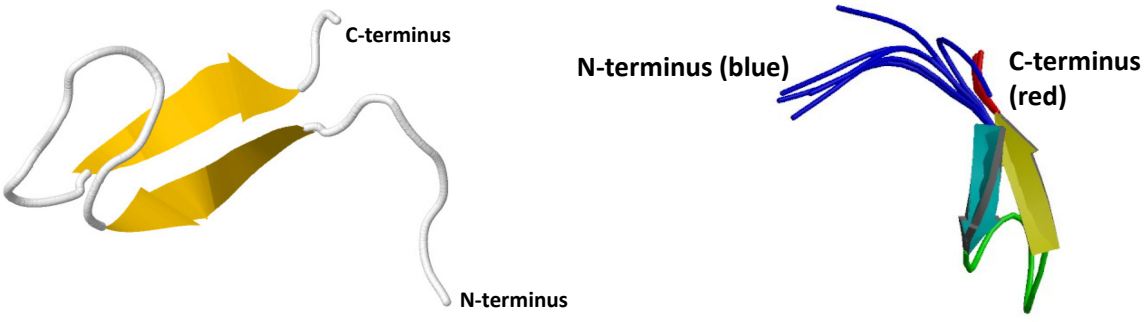

Figure 1.5: Solution NMR structure of human hepcidin-25 at 325K (PDB code: 2KEF), with residues 1-5 of the N-terminus not accurately assigned (different views) [86, 85].

It is of interest to be mentioned that the commercially available hepcidin-25 is obtained by synthesis of a linear 25-residue peptide, followed by oxidation of the eight cysteine-contained -SH groups, to form disulfide bridges of the folded peptide. Interestingly, the results of ion

mobility spectrometric (IMS) studies on two Hep-25 standards showed different conformers in terms of folding species, indicating the possibility of different hepcidin foldings achieved during synthesis [87].

1.2.2.2. Isoforms of hepcidin

In addition to Hep-25, two more N-truncated isoforms namely Hep-20 and Hep-22 were identified in urine by MALDI-TOF [39] (see *section 1.2.1.*). Later on, Kemna *et al.* confirmed using SELDI-TOF measurements that Hep-20 was present in both urine and serum, while Hep-22 was identified only in urine. Hence, the authors suggested that this isoform represented a urinary metabolite of Hep-25 [88]. Recently however, high resolution (HR) MS was able to detect Hep-20 and -22 in plasma, together with a novel hepcidin isoform, hepcidin-24 (Hep-24) [89, 90] (Figure 1.6).

In this context, several research groups investigated further the levels of the shorter hepcidin isoforms in serum relative to hepcidin-25 by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) and high resolution mass spectrometry (HR-MS). The results indicated relatively low level of the N-truncated isoforms compared to Hep-25 (Table 1.3). However, when cumulated, the isoforms could have an impact on the level of total hepcidin.

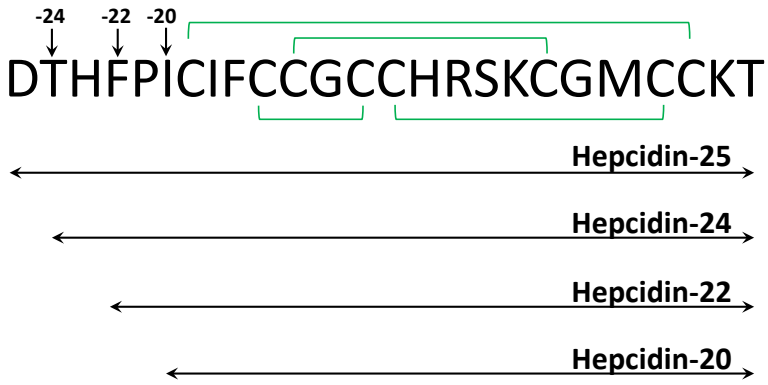

Figure 1.6: The known isoforms of hepcidin.

The bioactivity of these isoforms, evaluated as the ability to cause ferroportin internalization, was assessed by cellular studies. Experiments using synthetic N-truncated Hep-25 peptides [91] and mutated hepcidin-25 (alanine mutants in which each residue of the N-terminal region was individually replaced with alanine) [92] indicated that the lack of the N-terminal amino acids eliminates most of the bioactivity of the peptide (see *section 1.2.4.1.*). Laarakkers *et al.* performed cellular studies which confirmed the loss of activity for the shorter isoforms of Hep-25. The authors reported that Hep-24 presents a 10-fold lower activity compared to Hep-25, and the activities of both Hep-20 and Hep-22 were at least 10-fold lower than that of Hep-24. The

same study also showed that for long-term storage (0-7 days) of serum samples at room temperature, a decrease in Hep-25 was proportional to an increase in Hep-20, -22 and -24, indicating that the shorter isoforms represent serum breakdown products of hepcidin-25 [93].

Table 1.3: Serum level of hepcidin isoforms.

	Hep-25	Hep-24	Hep-22	Hep-20	Reference
Mean concentration (µg/L)	9	<LOQ (1 µg/L)	<LOQ (1 µg/L)	<LOQ (1 µg/L)	[94]
	12	N/A	0.97	2.6	[95]
	11*	N/A	N/A	3.9*	[96]

N/A = not available
**geometric mean*

1.2.3. Hepcidin-25-copper(II) complex

A particularity in the structure of hepcidin-25 is the presence of a small metal binding site called ATCUN (amino terminal Cu^{2+}- and Ni^{2+}-binding), identified in 2005 in the N-terminus of the peptide [97]. The affinity of hepcidin-25 for copper and the formation of a copper complex could be of notable importance for hepcidin analysis and its biological activity.

1.2.3.1. ATCUN motif – structure and importance

The ATCUN motif was first identified 50 years ago in human serum albumin (HSA) and has been studied intensively ever since. It depicts the presence of a metal binding site in the first three N-terminus residues of naturally occurring proteins. This small binding site is specific for the coordination of Cu^{2+} and Ni^{2+}. Moreover, the ATCUN motif consists of four nitrogen atoms that act as metal ligands. These involve the free α-NH_2 nitrogen, two intervening peptide nitrogen atoms and the imidazole nitrogen of a histidine residue in the third position. The crystal structure of the copper complex of Gly-Gly-His peptide shows the nitrogen atoms forming an approximately square planar geometry [98, 99]. Laussac *et al.* employed NMR spectroscopy to study the 24-residue N-terminus of BSA and suggested that the carboxylate group of the aspartate residue in the Asp-Ala-His motif may interact with the metal ion, forming a penta-coordinated structure [100]. This theory was however infirmed later by Hureau *et al.*, when the X-ray structure of the [Cu(II)(DAHK)] complex was revealed. The authors reported an axial coordination provided by a labile water molecule (Figure 1.7) and showed that Asp does not act as a fifth ligand [101]. Nevertheless, the importance of the aspartic acid in position 1 was attributed to the fact that Asp increases the basicity of the nitrogen atoms involved in the metal complex and thus the copper binding is enhanced [102].

Unfortunately, there is no available real crystal structure of the native protein-copper complex, probably due to the flexibility of the N-terminus in the native form of proteins and the difficulties encountered in the crystallization of large molecules. There are more than 25 structures of HSA reported on PDB, but none of these reveal the structure of the ATCUN motif. Nevertheless, it is generally accepted that this small metal binding site is included in the native form of the protein and, moreover, it is responsible for the action of albumin in transport of metals ions, including copper [98, 100].

In this context, other proteins were found to contain ATCUN motifs, with a preserved histidine residue in the third position. Additionally, the metal binding site was found to have a significant impact on their biological function. It has been reported that ATCUN motifs have been involved in DNA cleavage, gene targeting and protein design. As an example, the ATCUN site in human sperm protamine P2a may be responsible for inducing carcinogenesis in individuals exposed to metals [99, 103].

Moreover, copper binding sites located more central in proteins rather than at the flexible N-terminus and thus more accessible for structural and functional investigations, were shown to be responsible for catalysis, transport, detoxification and signaling [104]. Several enzymes and proteins such as cytochromes C oxidase, superoxide dismutase and metallothionein contain copper as a co-factor for their functional role in energy metabolism and antioxidant defense system. In this regards, ceruloplasmin, the main copper-containing protein, is a Cu^{2+}-dependent ferroxidase essential for iron mobilization [105] (see *section 1.1.1.1.*).

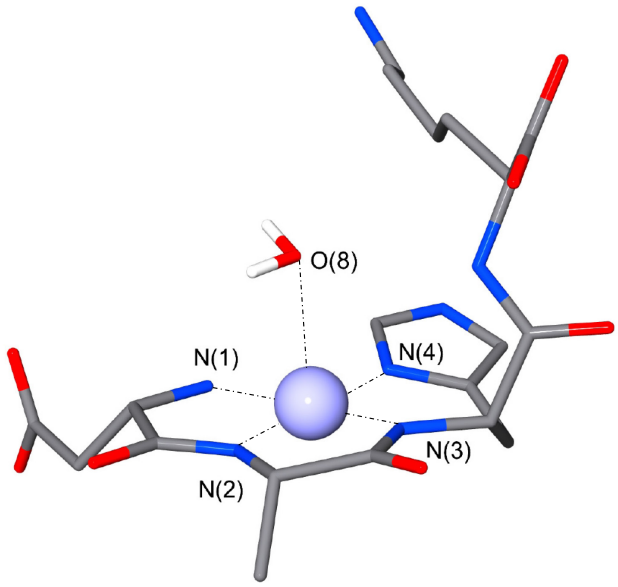

Figure 1.7: Crystal structure of [Cu(II)(DAHK)] [101] (CCDC — 809109 [106]) with a square-planar 4-N coordination geometry and an axial coordination of a labile H_2O molecule.

1.2.3.2. ATCUN motif in hepcidin-25

Melino *et al.* first confirmed the presence of the ATCUN motif in the structure of Hep-25 by circular dichroism (CD) and UV-Vis spectroscopy. The binding of Cu(II) to the N-terminal 9-amino acid fragment of hepcidin-25 yielded an absorption maximum at 525 nm in the pH range between 5.2 and 10.5. Moreover, the spectra of the whole hepcidin-25 in the presence of copper ions showed the presence of a maximum at 525 nm at a pH near neutrality [97].

Further, Tselepis *et al.* identified the ATCUN motif by HR-MS employing FTICR-MS (Fourier-transform ion cyclotron resonance MS). The ability of several transition metals (Cu^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+} and Ni^{2+}) to form complexes with human hepcidin-25 was tested. The authors reported no evidence of complex formation in the case of ferrous and ferric ions, while copper,

nickel and zinc ions all bound Hep-25 with a gradient of highest-to-lowest affinity as follows: $Cu^{2+} > Ni^{2+} > Zn^{2+}$ [107].

Tandem mass spectrometry (MS/MS) was also employed to characterize the Hep-25-copper(II) complex. Konz *et al.* applied collision induced dissociation (CID) which resulted in the fragment DTH-copper(II), representing the first three residues of the N-terminus of Hep-25 coupled to one copper ion. This indicated the stability of the copper-binding motif, which was preserved even during MS fragmentation [108].

Moreover, based on the affinity of Hep-25 for copper ions, immobilized metal ion affinity chromatography (IMAC) chips were applied for hepcidin purification (see *section 1.3.2.2.*) and ICP-MS (inductively coupled plasma mass spectrometry) was employed for hepcidin quantification (see *section 1.3.2.1.*).

Further, MALDI-TOF was used to characterize the interaction of Hep-25 to copper. A high affinity of the peptide hormone for copper was determined ($K_D=5\cdot10^{-7}$ M). Nevertheless, the authors concluded that this affinity is not sufficient for hepcidin-25 to compete with albumin ($K_D=10^{-12}$ M) [24] for the extracellular "free" copper [109]. However, a recent study by Plonka *et al.* employing potentiometric titration and UV-Vis spectroscopy, which are widely recognized as the standard procedure for binding constant calculations [110, 111], characterized the flexible N-terminal hexapeptide of hepcidin-25 (DTHFPI) as the strongest ATCUN motif ever reported with a dissociation constant of $10^{-14.66}$ M. Moreover, this high affinity for Cu(II) ions was confirmed by a quick transfer of Cu^{2+} from HSA to this peptide. Based on these results, the authors proposed that hepcidin-25 exists, at least sometimes, as Cu(II) complex in blood, and stated that hepcidin-25-copper(II) complex warrants a continued research [112].

1.2.3.3. Copper homeostasis

The copper ions required for body needs arise generally from diet. The average dietary copper intake in adults is 0.6-1.6 mg/day. Copper is mainly contained in shellfish, organ meats and seeds. Relatively smaller amounts are also found in vegetables, muscle meats and water.

Copper trafficking is a complex process influenced by both metal-protein binding constants and by the use of copper in biological processes [113, 105]. Once absorbed in the small intestine (mainly as Cu^{2+}), copper is transferred to the blood stream where it can bind human albumin (HSA), which exhibits a high affinity for Cu^{2+} due to the presence of the ATCUN motif. However, only ≈ 180 µg of copper are incorporated by HSA, even though albumin has the capacity to accommodate approximately 40 mg of copper. The rest of Cu^{2+} (≈1 mg) is contained in ceruloplasmin (65%), transcuprein (12%) and other low molecular weight components such as peptides and amino acids (≈20-50 µg) [105]. Interestingly, ceruloplasmin does not bind copper directly in the presence of the metal ions, rather 6-7 molecules of Cu^{2+} are added during its synthesis in the liver. The lack of copper during the synthesis of ceruloplasmin leads to an unstable protein and the loss of its activity as ferroxidase (see *section 1.1.1.1.*).

A “free” extracellular copper concentration of 1-2 µM (unbound fraction) was reported by McMillin *et al.*, that was obtained after separation from the bound fractions of copper through ultrafiltration, using a molecular weight cutoff of 30 kDa, which should eliminate albumin, ceruloplasmin, and other proteins expected to bind copper [114].

1.2.4. Hepcidin-25 activity

The essential role of hepcidin in systemic iron homeostasis has been established by animal models, shortly after its discovery in 2001 (see *section 1.2.1.*). Therefore, considerable efforts have been made ever since for the elucidation of the mechanism of hepcidin-25 activity as the iron regulator and the factors implicated in hepcidin-25 regulation.

1.2.4.1. The mechanism of action of hepcidin-25

Hep-25 interrupts iron release into the blood stream from the three main sources of iron in the body: dietary iron absorption by the enterocytes, iron recycling by the macrophages and stored iron mobilization by the hepatocytes [35] (see *section 1.1.2.,* Figure 1.8a). Studies on cultural cells expressing mouse FPN (cell line HEK293-Fpn) with a C-terminal green fluorescent protein (GFP) confirmed that hepcidin binds FPN, the 571-residue protein known as the sole iron exporter (see *section 1.1.2.*), causing its internalization and degradation. Concentrations of 100 nM hepcidin-25 caused ferroportin internalization within 1 hour, while a 10 nM solution of hepcidin-25 led to ferroportin internalization within 3 hours [40]. In this context, the physiological level of hepcidin-25 in human adults is around 3.5 nM (10 µg/L) (see *section 1.3.3.3.).*

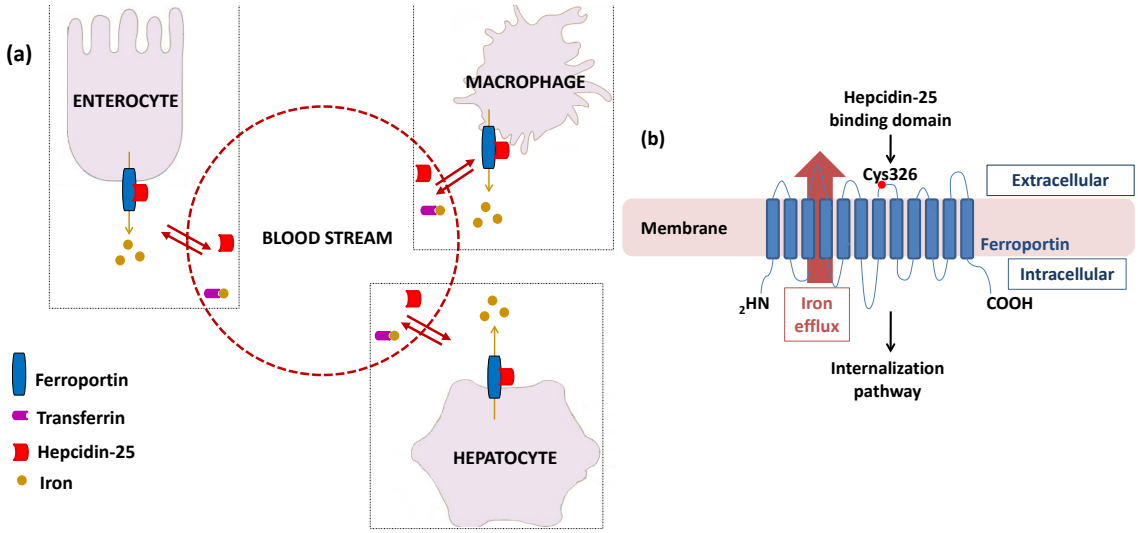

Figure 1.8: (a) Iron homeostasis regulated via hepcidin-25 by binding FPN in the three iron releasing cells: enterocytes, macrophages and hepatocytes; (b) Hep-25 – FPN interaction [115].

Further studies by Nemeth *et al.* using the same cell line, measuring the ability of hep-25 to induce ferroportin endocytosis, revealed that the lack of the five N-terminal amino acids restricts the biological activity of the peptide [91]. Clark *et al.* also investigated the importance

of the N-terminus for the interaction with FPN by substituting the N-terminus individually with alanine (residues 1–6 and 8). The authors reported that His3, Phe4 and Ile6 were significant for Hep-25 activity [92]. Moreover, mini-hepcidins consisting of the first nine N-terminal residues were designed and tested for their iron regulation ability. They induced hypoferremia in mice, showing even superior bioactivity compared to native Hep-25 [116].
Recent studies employed by Fernandes *et al.* on the down-regulation of FPN by Hep-25, using similar HEK293 cell line with GFP showed the implication of the extracellular residue Cys326 of FPN in Hep-25 binding [117] (Figure 1.8b). The authors hypothesized that the Hep-25 – FPN interaction is related to a disulfide bond formation or rearrangement involving the -SH group of the Cys326 residue of FPN and the disulfide bridge of Hep-25. Additionally, the interaction was not affected by modulation of an enzyme implicated in disulfide rearrangement (thiol isomerase), but was sensitive to reducing agents, indicating that the binding could be associated with a non-enzymatic disulfide rearrangement.

1.2.4.2. Hepcidin-25 regulation

Each of the stimuli known to alter iron homeostasis affect hepcidin expression as well. Hence, Hep-25 synthesis is stimulated in response to iron overload and inflammation and is down-regulated by iron deficiency, increased erythropoietic activity and hypoxia [22]. However, the signaling pathways by which these stimuli affect hepcidin expression are not fully elucidated. It seems that the key signaling pathway for the regulation of Hep-25 expression is the BMP/SMAD pathway with haemojuvelin HJV as cofactor. The bone morphogenetic proteins (BMPs) represent growth factors also known as cytokines, involved in many processes such as bone development or tissue repair and formation. In the liver, the BMP pathway seems to be adapted specifically for iron regulation via a combination of cofactors including HJV [56, 35]. In this regard, the regulation of hepcidin expression via iron or BMP pathway leads to low levels of Hep-25 in HH type 1-3, owing to mutations of HFE, HJV, TfR and HAMP genes (see *section 1.1.3.2.*).
Additionally, Hep-25, like other hormones, is regulated by the substance whose levels it controls, namely iron. Hypoferremia and iron overload regulate hepcidin synthesis and excretion by the hepatocytes. The transferrin receptors (TfR1 and TfR2) and the membrane protein HFE (see *section 1.1.3.2.*) seem to be involved in this process [35].
Another signaling pathway for Hep-25 transcription, activated in inflammatory conditions, is represented by the signal transducer and activator of transcription 3 (STAT-3) which is regulated by interleukin-6 (IL-6) and possibly other inflammatory cytokines. The increase of inflammation-induced Hep-25 is responsible for the hypoferremia that occurs in inflammatory diseases (see *section 1.1.3.1.*) [35, 118].
Recently, MT-2 was discovered as a novel regulator of hepcidin transcription which down-regulates the HAMP expression following iron deprivation [119]. This explains the reason for

which a mutation of the gene encoding MT-2 (TMPRSS6) in IRIDA causes the stimulation of Hep-25 production (see *section 1.1.3.1.*).
Finally, a new hormone called erythroferrone was identified in 2014. It is produced by erythroblasts in response to erythropoietin, and mediates hepcidin suppression during stress erythropoiesis [120].

1.2.5. The clinical value of hepcidin-25

Several recent reviews (2013-2017) on hepcidin-25 highlight the potential of the peptide not only as a clinical parameter for the diagnosis and prognosis of iron-related disorders [121, 76, 122, 123], but also as a therapeutic target [71, 124-126].

1.2.5.1. Hepcidin-25 – a promising biomarker in iron pathology

The ability of body iron pools to change independently from each other makes the "iron status" a complex process that can vary significantly. In this respect, there is no single laboratory parameter that provides full information about iron metabolism [127]. Currently, several iron status indicators are clinically available and a combination of these parameters is used for the diagnosis of iron disorders. The most common biomarkers tested in iron deficiency are ferritin (Ft), soluble transferrin receptors (sTfRs), transferrin saturation (TSAT) and reticulocyte hemoglobin content (CHr). These are indicators for body iron stores (Ft), tissue iron deficiency (sTfRs), circulating iron (TSAT) and/or iron demand for erythropoiesis (CHr). However, the explosion of knowledge of iron metabolism regulated by Hep-25 make the clinicians uncertain, which approach to diagnose iron disorders is correct. Hepcidin-25, as the main regulator of systemic iron homeostasis, has direct influence on these parameters and can be a suitable candidate for a comprehensive iron indicator [128, 122]. An advantage of Hep-25 over the conventional biomarkers is the "real-time" information of iron status provided. This is because the production of hepcidin is substantially increased within a few hours after iron administration [129, 130].
Most of the iron disorders were revised in the hepcidin era and were linked to hepcidin-25 (see *section 1.1.3.*). Level fluctuations of this peptide hormone are either the cause or the effect of iron overload or hypoferremia (Table 1.4). In this sense, Hep-25 can be particularly useful in differentiating distinct types of hypoferremia associated for example with IDA and ACD. The hypoferremia that occurs in IDA when iron stores (usually from the liver) are depleted, is compensated by low levels of hepcidin-25 that would allow any absorbed iron in the enterocyte to be released in the circulation. On the other hand, the inflammation in ACD caused by IL-6 stimulates the production of hepcidin-25 that leads to the blockage of iron export resulting in hypoferremia (see *section 1.1.3.1.* and *section 1.2.4.2.*).
In 2002, Thomas *et al.* introduced a diagnostic plot correlating the ferritin index (sTfR/log ferritin) and CHr, as an alternative to the conventional markers used in the diagnosis of iron deficiency [131]. The authors showed that this so-called Thomas plot improved tracking the ID

progression. In 2011, the same authors tested the possibility to replace ferritin index with hepcidin-25 in the Thomas plot, emphasizing the potential of the peptide to discriminate between states of anemia [127].

Table 1.4: Hep-25 plasmatic levels in various iron disorders [69].

	Disorder	Regulation of hepcidin expression	Fluctuation of Hep-25 and Fe plasmatic levels
Iron overload	HH 1-3	Mutation of HFE, HAMP, HJV, TfR	Hep-25 ↓ → Fe ↑
	β-Thalassemia	Defective erythropoiesis	Hep-25 ↓ → Fe ↑
Iron deficiency	ACD	IL-6-mediated inflammation	Hep-25 ↑ → Fe ↓
	IDA	Depletion of iron stores	Fe ↓ → Hep-25 ↓
	IRIDA	Mutation of TMPRSS6	Hep-25 ↑ → Fe ↓
	Anemia of CKD	IL-6-mediated inflammation	Hep-25 ↑ → Fe ↓

Numerous assays for the quantification of Hep-25 in biological samples have been developed (see *section 1.3.*), however, there is a lack of a reference method that could permit the use of Hep-25 as a clinically accepted parameter. In this vein, efforts towards worldwide hepcidin-25 harmonization are ongoing [132] (see *section 1.3.3.*).

Nevertheless, many relevant authors to the iron-related scientific community endorsed hepcidin-25 as a promising biomarker in the clinical field, with applications covering particularly the diagnosis of iron overload, diagnosis and management of IDA (especially IRIDA), differentiation between ACD and IDA or ID monitoring and therapy in anemia of CKD [69, 76].

1.2.5.2. Hepcidin-25 therapy

Since Hep-25 excess or deficiency influences the pathogenesis of iron disorders, Hep-25 agonists and antagonists could improve the therapy applied for these diseases. Hepcidin deficiency is prevalent in most types of hemochromatosis (HH type 1-3) and in β-thalassemia. Hence, modulation of hepcidin in these patients to correct the iron overload could increase the patient compliance by replacing blood transfusions (see *1.1.3.2. section*). Mini-hepcidins were designed in this regard and were shown to reproduce Hep-25 activity in mice [116]. Such hepcidin mimetics were further developed by pharmaceutical companies to be used in therapy of iron overload and are in Phase I clinical trial (M012 by Merganser Biotech and LJPC-401 by La Jolla Pharmaceuticals Company). Additionally, hepcidin-25 agonists as stimulators of hepcidin-25 production by targeting the BMP pathway and TMPRSS6 gene were also suggested [71, 133].

Increased levels of hepcidin-25 are associated with ACD, IRIDA and anemia of CKD (see *section 1.2.4.1.*). The current treatment available includes ESAs (see *section 1.1.3.1.*), however, erythropoietin resistance is frequently observed as a consequence of inflammation [134]. In this respect, Hep-25 antagonists could increase available iron to correct for anemia. Multiple strategies can be applied on this account, such as decreasing hepcidin production with BMP antag-

onists or IL-6 inhibitors or neutralizing the peptide with anticalins and spiegelmers. Pieris Pharmaceuticals developed a bioengineered lipocalin that captures hepcidin to prevent FPN degradation, which is in Phase I clinical trial. In this context, the most advanced Hep-25 antagonist is developed by Noxxon Pharma AG. NOX-H94, a L-RNA spiegelmer hepcidin inhibitor, is in Phase II clinical trial and is designed to antagonize the Hep-25-induced FPN degradation [71, 133, 124]. Amgen and Eli Lilly and Company developed humanized monoclonal antibodies for capturing hepcidin. Eli Lilly's anti-hepcidin antibody (LY2787106) finished the Phase I clinical trial, but was subsequently replaced by an anti-FPN antibody (LY2928057), that obstructs hepcidin binding but not FPN function. Concurrently, the anti-hepcidin antibody developed by Amgen (12B9m) is reported to be in preclinical development [71, 133].

1.3. Analytical strategies for the quantification of hepcidin-25

The illustration of the clinical value of hepcidin-25 triggered significant efforts for the development of a robust assay to be implemented in clinical laboratories for the quantification of this iron-regulator in biological fluids. Mainly, two types of strategies were applied for the clinical assessment of hepcidin-25: immunological and mass spectrometry-based methods.
Initially, semi-quantification assays for hepcidin-25 in urine samples were developed, employing both antibody-based methods [118] and mass spectrometry [135]. Comparative studies of urine and serum samples showed that on one hand urinary hepcidin seems more affected by multiple freeze-thaw cycles, but on the other hand serum hepcidin is more influenced by diurnal variation. However, the urinary determination of Hep-25 requires further quantification of creatinine, a parameter characterizing the renal clearance. Creatinine is used for the concentration normalization of most of the urinary parameters by adjusting alterations in urine concentrations. Thus, urinary assays could be hampered in the patients with renal diseases. In light of this, most of the quantification assays focused on analysis of the peptide hormone in serum samples. Additionally, several studies showed no differences in the hepcidin-25 levels in human serum relative to plasma [136, 93]. Taking this into consideration, an overview of the analytical techniques which focused on serum hepcidin-25 quantification will be highlighted in the next sections, underlining their general clinical usage and their potential for routine analysis.

1.3.1. Immunoassays

Principle of immunoassays

In 1959, Yalow and Berson first reported the principles of immunoassays [137], a bioanalytical tool based on a response signal generated from an antibody-antigen interaction. Antibodies, also known as immunoglobulins (Ig), are proteins produced by the immune system to recognize and neutralize foreign substances (antigens) entering the body. The antibody presents a paratope or antigen-binding site that specifically binds the recognition site of the antigen called epitope. The response signal from the antibody-antigen reaction is produced by label (e.g. enzymatic,

radioactive, fluorescent or luminescent) attached to the antigen (analyte) or the antibody, and permits the quantitation of the analyte [138]. Mainly, two types of antibodies are used in immunoassays, namely mono- and polyclonal antibodies. A polyclonal antibody represents a collection of antibodies from different antibody secreting B cells that recognize multiple epitopes, while a monoclonal antibody is secreted by a single B lymphocyte and binds with one unique epitope. There is a multitude of immunoassay formats available, for example with immobilized antigen or immobilized antibody on a solid surface, heterogeneous or homogeneous, competitive or non-competitive.

Immunoassays have wide applications in pharmaceutical analysis, medical research and routine analysis. The firstly established assays were radio-immunoassays (RIA), however, strict regulatory controls of the radioactive labels have favored the exclusion of RIA from the routine analysis, and their replacement with enzyme immunoassays (EIA), which use enzyme-labeled conjugates. One of the most popular EIA is enzyme-linked immunosorbent assay (ELISA). With a high throughput, low costs involved and high sensitivity for a wide range of analytes, EIA, especially ELISA, has become a reference method for medical laboratories, regulatory bodies or proficiency tests organizations [139]. However, a major limitation of immunoassays is the development of suitable antibodies against the desired analyte. In this respect, certain characteristics of antibodies, such as affinity (the ability to form antibody-antigen complex) or cross-reactivity (interactions of the antibodies with other compounds than the desired analyte) are often not sufficiently investigated, leading to misinterpretation of the results [140].

Immunoassays in hepcidin-25 analysis

Immunoassays were the first analytical techniques developed for the quantification of hepcidin in biological samples due to their high potential for developing into a reference method for routine laboratories. Several formats were tested in this regard.

The first immunoassay, developed by Ganz *et al.*, was a competitive ELISA using biotinylated hepcidin-25 as tracer [141]. An alternative ELISA was also developed using recombinant tagged peptide (hepcidin-25-His) [142]. However, both techniques employed polyclonal antibodies which were unable to differentiate sufficiently between different hepcidin isoforms. A monoclonal antibody was developed, however, its cross-reactivity relative to the other isoforms was not tested [143]. Nonetheless, a selective sandwich ELISA for Hep-25 was developed by Eli Lilly and Company employing a monoclonal antibody that showed no cross-reactivity to Hep-20 and Hep-22 [144]. Although the newly discovered Hep-24 was not tested at that time, the method assured a sensitive and robust hepcidin-25 quantification, with a reported LOQ of 0.01 μg/L. Unfortunately, the developed antibody is not commercially available.

Grebenchtchikov *et al.* developed a radioimmunoassay for hepcidin-25 quantification using a polyclonal antibody and ^{125}I-labeled hepcidin [145]. Because of several disadvantages of RIA such as short shelf-life conjugates, complex equipment and highly trained personnel needed due to medical hazards, the aforementioned research group later developed a competitive ELISA using the same polyclonal antibody and biotinylated hepcidin-25 as a tracer [146]. This

method is currently used for total hepcidin measurements in the frame of the hepcidinanalysis.com initiative on a fee-for-service basis.

At this point, it can be concluded that the limited availability of suitable antibodies for the selective determination of bioactive hepcidin-25 hinders the applicability of immunoassays for the quantification of the iron regulator. Immunoassays are suitable for the measurement of total hepcidin, however, since the correlation between Hep-25 levels and the other isoforms is not fully elucidated, the results can be difficult to interpret.

On this matter, there are several reasons that limit the development of a reliable immunoassay for hepcidin-25. Development of a selective antibody for the bioactive isoform is hampered by the small size of the molecule that reduces the number of antigenic epitopes, and by the structural similarities to the other isoforms, which could be relevant cross-reactants (see *section 1.2.2.2.*). Additionally, Hep-25 is an evolutionarily conserved peptide. Therefore, the development of a monoclonal antibody that implies the use of mice could be hindered by the similarity of amino acid sequences in humans and mice. Also, the shortage of suitable hepcidin-25 conjugates to be used as tracers could cause lack of reliable immunoassays. Reason for this is that the compact structure of Hep-25 and its tendency to aggregation, especially around its isoelectric point of 8.2, make difficult the handling of the peptide at adequate pH values for bioconjugation (generally around 7.4-8.5 [147, 148]).

In this context, mass spectrometry (MS) could provide an alternative to immunoassays for hepcidin-25 quantification. MS-based techniques represent an emerging tool for the clinical assessment of proteins and small peptides in biological fluids due to high sensitivity and selectivity. Additionally, these methods can be developed in a relatively short period of time compared to immunoassays, where the development of affine monoclonal antibodies can last years.

1.3.2. Mass spectrometry based methods

Mass spectrometry (MS) is a technique that dates back more than a century, with a continuously developing technology applied initially solely in physics and chemistry, and, in the past two decades, extended to biology, biochemistry and biomedical research [149]. In the field of laboratory medicine, mass spectrometry is now generally considered as a fundamental tool for "next generation clinical chemistry" [150].

MS provides qualitative and quantitative information about chemical species after conversion to ions in the ionization source and subsequent separation of the obtained ions in the mass analyzer based on their mass-to-charge ratio (m/z). Ion signals are generated in the detector and recorded in a computer system as a function of the m/z ratio, generating the mass spectrum [151].

Mass spectrometry includes organic (molecular) MS used for analysis of (bio)molecules, and inorganic (elemental) MS, also known as inductively coupled plasma (ICP) MS, applied for the detection of metals and several non-metals. Molecular MS commonly refers to electrospray (ESI) MS and matrix assisted laser desorption/ionization (MALDI) MS. ICP-MS, MALDI-MS

and ESI-MS were employed for hepcidin-25 quantification. Suitability of these techniques for hepcidin-25 assessment in clinical samples will be evaluated in the following sections.

1.3.2.1. ICP-MS

Principle of operation

Inductively coupled plasma mass spectrometry (ICP-MS), developed in the early 1980s, is a sensitive analytical tool for elemental analysis [152]. ICP-MS uses a plasma discharge at very high temperatures to generate positively charged ions. In this respect, the sample is first inserted into the sample introduction system, consisting of the spray chamber and the nebulizer. Then, an aerosol is generated, that reaches the base of the plasma through the sample injector. The sample is further dried, vaporized, atomized, and ionized through the different heating zones of the plasma torch. This means that the liquid aerosol is transformed first into solid particles, then into a gas. Subsequently, at approximately 6000–7000 K in the analytical zone of the plasma, the sample exists as excited atoms and ions, which provides the elemental composition of the sample [153].

An advantage of ICP-MS is the possibility to analyze gaseous, liquid and solid samples, due to the elevated temperatures of the plasma that provide an ideal atomizer and element ionizer for all types of samples and matrixes. Characterized by a high sensitivity (ppt), ICP-MS is considered the "gold standard" method for ultra-trace elemental analysis [152].

Hepcidin-25 quantification

Konz *et al.* exploited this technique for the assessment of hepcidin-25 in serum samples by employing direct biomolecule labeling. The affinity of hepcidin-25 for Cu(II) due to the presence of the ATCUN motif (see *section 1.2.3.*) permitted the quantification of the peptide by determining the amount of copper binding to hepcidin-25 using LC-ICP-MS. Anion exchange chromatography was employed for separation of the peptide-copper(II) complex and the excess of metal [108]. However, the limited stability of these columns hinders a robust and reproducible quantification. Also, the mass ratio Hep-25:Cu^{2+} of approximately 43:1 determined an equivalent decrease in the limit of detection (LOD) for the peptide analysis. The very good sensitivity of the elemental MS achieved for copper (LOD 33 ng/L) corresponds to a LOD for hepcidin-25 of 1.4 µg/L and a reported LOQ of 1.8 µg/L. In contrast, ESI-MS provides quantification options that are more sensitive, as it will be presented in *section 1.3.2.3.* Additionally, ICP-MS provides no structural information on the biomolecule, hence there is no certainty that the quantified metal arises exclusively from the biomolecule complex. Because of this, the clinical quantification of biomolecules using ICP-MS based on the analysis of their complexes with metals is debatable. Generally, ICP-MS is applied for trace analysis of metals (and some non-metals) in environmental analysis, food analysis or forensics. However, for peptide quantification in clinical chemistry, ESI-MS is the preferred MS-based technique due to high specificity and sensitivity [150].

1.3.2.2. MALDI-MS

MALDI-MS - Principle of operation

Matrix-assisted laser desorption/ionization (MALDI), established by Karas and Hillenkamp in 1988 [154], is a soft ionization technique consisting in the irradiation of an analyte-matrix mixture with a laser, with minimal fragmentation. Here, the analyte of interest is co-crystallized on a metal plate (target) with a high molar excess of matrix. In this vein, the dried droplet approach is most commonly used, where droplets of matrix and sample are placed on the MALDI target and dried at room temperature. Typically, the matrix is a weak organic acid (e.g. α-cyano-4-hydroxycinnamic acid, 2,5-dihydroxybenzoic acid, sinapinic acid), characterized by an absorption wavelength correspondent to the laser wavelength (usually in the ultraviolet range). Commonly, nitrogen lasers (337 nm) and frequency-tripled and quadrupled Nd:YAG lasers (355 nm and 266 nm respectively) are employed for this purpose [155]. Further, the matrix strongly absorbs the laser radiation energy, causing its vaporization and, indirectly, the vaporization of the analyte. Also, the matrix contains additives that can improve the co-crystallization, sequestrate the excess of salts and function as H^+ donors (e.g. trifluoroacetic acid TFA), which leads to the ionization of the sample analyte [156].

MALDI-MS provides molecular weight information on intact analytes with high accuracies in the low-ppm range. Therefore, MALDI-MS is an excellent analytical tool for peptide and protein identification and characterization. This technique is thus mainly used for qualitative purposes, with limited applicability for quantitative analysis due to poor shot-to-shot repeatability caused by the inhomogeneity of the analyte-matrix mixture and laser fluctuations [155].

SELDI-MS

A distinct type of laser desorption/ionization mass spectrometry is surface enhanced laser desorption/ionization (SELDI) MS, established in 1993 by Hutchens and Yip [157]. SELDI can be considered a variation of MALDI that differs in the sample preparation. In this regard, the analyte of interest (usually proteins) is applied to a modified surface (chip) that plays an active role in the extraction, purification or modification of the analyte prior to desorption and ionization for MS analysis. A variety of materials can be used for this purpose, such as metals, cation/anion exchangers, hydrophobic compounds similar to C8 reverse phase chromatography and others [158].The samples spotted on the MALDI target or the SELDI surface are subjected further to MS analysis, typically using time-of-flight (TOF) mass analyzers, where the ions are separated based on their velocity differences. The m/z ratio is further determined by measuring the time needed for the formed ions to reach the detector.

SELDI/MALDI-MS quantification of hepcidin-25

SELDI-TOF-MS was the first MS method employed for the semi-quantification of hepcidin-25 in serum, using immobilized metal ion affinity chromatography (IMAC) chips [159], based on the affinity of hepcidin-25 for copper (see *section 1.2.3.*). Later, Swinkels *et al.* utilized similar IMAC chips in an improved SELDI-TOF quantification method using hepcidin-24 as internal standard [160]. However, different biochemical characteristics of the internal standard due to

the lack of aspartic acid caused different binding behavior to the IMAC chips and hampered a robust quantification assay. Weak cation exchange (WCX) beads were further investigated as an alternative modified surface by the same group. Also, with a better mass resolution, MALDI-TOF "replaced" the SELDI-TOF analysis. This methodology was characterized by a coefficient of variation CV < 10% and LOD of 1.5 µg/L [146]. Furthermore, Anderson *et al.* used ready-to-use MALDI chips with vacuum sublimated, ultra-fine homogeneous matrix composition that provided improved spot-spot reproducibility over the classic dried droplet approach [161]. A good precision was achieved (<15%), however, the method proved to be less sensitive with a reported LOQ of 2.8 µg/L.

Additionally, commercially available hepcidin-25 measurements are available in the frame of the hepcidinanalysis.com initiative. WCX-MALDI-TOF with isotope enriched hepcidin-25^{+40} as internal standard is applied for this purpose, with a reported LOQ of 1.4 µg/L [162, 93].

However, even though MALDI-TOF offers high throughput desired in routine laboratories, there are several disadvantages that restrict its use for absolute quantification in clinical field, such as the non-linear dependency of signal intensity and analyte concentration, with a dynamic range usually limited to less than two orders of magnitude. Also, the heterogeneity of analyte-matrix deposits and laser fluctuations hinder reproducible data acquisition [163]. Moreover, many MALDI-MS systems do not offer tandem mass-spectrometry (MS/MS) capabilities, determining limited selectivity compared to LC-ESI-MS/MS. On the other hand, isotope dilution (ID) LC-MS/MS (see *section 1.3.2.3.*) is accepted as the MS "gold standard" for quantification of biomolecules in clinical chemistry, providing high selectivity and excellent precision and accuracy [164].

In this context, the clinical assessment of hepcidin-25 is a case of quantification of a low abundant analyte in complex matrixes (serum/plasma samples). Thus, the best choice for the quantification of this analyte in biological fluids is LC-ESI-MS/MS with clearly superior specificity and reproducibility over MALDI-MS.

1.3.2.3. ESI-MS, LC-(ESI)-MS/MS

Advances in MS instrumentation, such as the development of electrospray ionization (ESI) source [165], the direct coupling of liquid chromatography to mass spectrometry (LC-MS) [166] or the coupling of two mass analyzers in tandem mass spectrometry (MS/MS) techniques [167], made mass spectrometry occupy a leading role in the field of clinical diagnostics, with applications in structural analysis and quantification measurements. LC-ESI-MS/MS is the most common mass spectrometry-based quantification method used in clinical laboratories nowadays [168], as an emerging alternative to immunoassays. An example in this regard is the quantification of steroid hormones in biological fluids, where LC-MS/MS is widely accepted as the preferred method, over immunoassays [169]. Also, reference quantification methods for the quantification of folates [170] and small endogenous metabolites [171] using LC-MS/MS were established. Further discussion on the principle of ESI, MS/MS and LC-ESI-MS/MS and

their applications in hepcidin-25 quantification is provided next, together with the pitfalls associated and their troubleshooting.

ESI - principle of operation

Electrospray ionization, established by Yamashita and Fenn in 1984 [165], is a soft, atmospheric pressure ionization technique, that occurs in liquid phase and is commonly applied to (polar) analytes in various polar solvents. Thus, ESI can be used for a wide range of molecules, including non-volatile, thermally labile biomolecules, that cannot be analyzed using more conventional techniques such as atmospheric pressure chemical ionization (APCI) or electron impact ionization (EI) [151].

Generally, ESI-MS can be divided into three steps: nebulization of a solution into electrically charged droplets with generation of a fine spray, evaporation of solvent with subsequent delivery of ions from droplets and transportation of the ions from the atmospheric pressure ionization source area into the vacuum of the mass analyzer [172, 173]. First, the sample solution is introduced into a stainless steel or quartz vessel, called capillary, which ends in a very fine tip. Then, a high voltage (2-6 kV) is applied between the capillary tip and a counter-electrode which is located at the entrance of the mass analyzer, generating a mist of highly charged droplets with the same polarity as the capillary voltage (Figure 1.9). When the electrostatic repulsion of the charges overcomes the surface tension (Rayleigh limit), charged micro-droplets are released from the surface towards the counter-electrode [172]. In this vein, increased temperature and a nebulizing gas (e.g. nitrogen) assist the reduction in size of the charged droplets. Further, the droplet radius decreases progressively, while the surface charged density increases [151], followed by the emission of the ions from the highly charged micro-droplet. Two models were proposed in this regard. Iribarne *et al.* suggested that when the strength of the electric field at the surface of the droplet is high enough, ions may be emitted directly from the highly charged droplet to the surrounding gas due to Coulomb repulsion forces (Ion Evaporation Model) [174]. A second theory by Dole *et al.* [175], supported later by Schmelzeisen-Redecker *et al.* [176], claims that repetitive Coulomb explosions lead to ion separation from the surface of the droplets as nano-droplets, released by de-solvation of the remaining solvent (Charged Residue Model). Either way, “naked” sample ions are finally generated, which are sampled by a sampling skimmer cone and further transported in the mass analyzer [172].

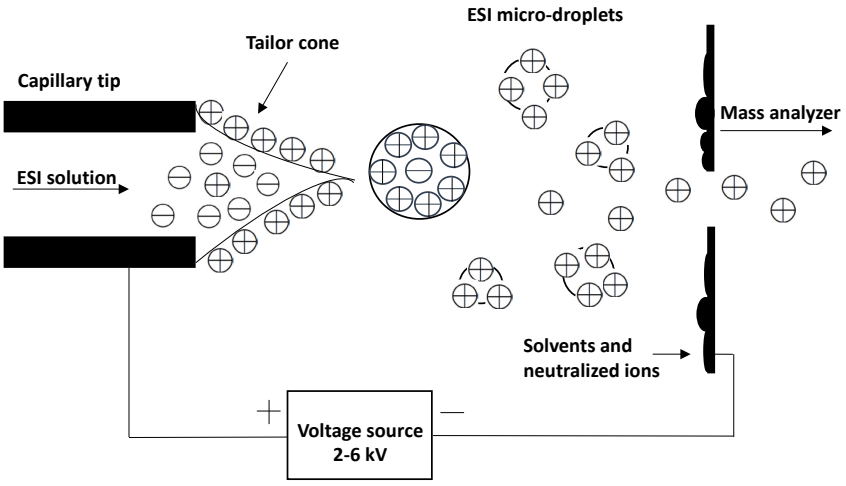

Figure 1.9: Schematic of the ESI process (positive mode) [177].

Ionization process

There are diverse ways to obtain sample ions in solution for ESI-MS analysis. Molecular ions are formed by the removal from (M^{n+}) or addition to (M^{n-}) a molecule of one or more electrons [178], where n represents the number of charges per ion. However, this is rarely the case in ESI. Usually, the molecule is protonated ($[M+nH]^{n+}$) or deprotonated ($[M-nH]^{n-}$), in which case the ions are named quasi-molecular ions. Consequently, the mass range of any mass analyzer is characterized by a n-fold enhancement. Therefore, compounds with high molecular masses such as proteins can be identified using ESI-MS at relatively small m/z ratios [179].

Some compounds already exist as ions in solution, for example quaternary ammonium salts or salts from strong acids (sulfates, phosphates). In other cases, the pH needs to be adjusted either to protonate a base or deprotonate an acid. Hence, the ionization of amines such as peptides or proteins requires acidic pH, whereas basic pH is employed for ionizing acidic samples such as carboxylic acids or phenols. However, protonation and deprotonation cannot be applied for other polar compounds that do not contain basic or acidic groups. Alternatively, these compounds can be associated with other ions in solution [172]. Usually, sodium and potassium ions are used for the detection of a sample as positive ions $[M-nH+mNa]^{(m-n)+}$ or $[M-nH+mK]^{(m-n)+}$, due to their affinities for N-methylacetamide anion [180]. Negative ions such as acetate, formate or trifluoroacetate are used for the detection of a sample as negative ions $[M+nH-mX]^{(m-n)-}$. Examples of such compounds are amides, esters, ethers or carbohydrates [172].

For pH control, volatile bases, such as ammonium hydroxide, or volatile acids, such as formic acid (FA), trifluoroacetic acid (TFA) or acetic acid, are used. In high-performance liquid chromatography (HPLC), these reagents are commonly added in the mobile phases as ion-pairing agents to improve chromatographic resolution. Therefore, HPLC devices can be easily hyphenated online to ESI sources, when compatible flow rates are applied. However, the ion-pairing reagents can be a source of ion suppression in MS. For example, the use of TFA is controversial. There are studies showing strong suppression of the ionization efficiency of peptides using TFA in some ion sources [181]. In this vein, Kuhlmann *et al.* developed methods to reduce the

signal suppression of strong acids such as TFA by post-column addition of TFA-fix mixtures containing isopropanol and propionic acid [182]. On the other hand, some ion sources are not significantly affected by the use of TFA, even over long periods of time [183]. Generally, TFA-based ion suppression influences strong basic compounds. The cause is intense ion pairing between the TFA anion and the protonated cation of basic compounds. The protonated cations are "camouflaged" and appear neutral to the electric fields of the ionization source. However, weakly basic molecules are not significantly affected by this process [182].

Nevertheless, signal suppression of the analytes of interest cannot be attributed to one cause only such as the ion-pairing reagents used as additives, but it is a complex process dependent on many factors. Signal suppression (or enhancement), defined as the variability in the ionization response of the target compound, depends on the chemical properties of the target molecule, the pH of the solution, the concentration of electrolytes and other matrix components, the clean-up protocol of the sample, the chromatographic conditions, including both stationary and mobile phases, and the MS instrumentation (source design, ionization mode) [184, 185]. In case of complex clinical samples such as plasma or serum samples, it was shown that ionization suppression is mainly caused by non-volatile materials from the biological sample extracts [185].

The sensitivity of ESI-MS is highly influenced by the ability to produce gas-phase ions from the molecules of the analyte in solution (ionization efficiency) and the capability to transfer the formed ions from atmospheric pressure to the vacuum area in the mass analyzer (transmission efficiency). The ionization efficiency, defined by IUPAC as "the ratio of the number of ions formed to the number of electrons or photons used in an ionization process" [178], can be affected by several factors such as flow rate, interface design, solvent properties (polarity, pH) or analyte characteristics (basicity, polarity, volatility, molecular size). The transmission efficiency, defined as "the ratio of the number of ions leaving a region of a mass spectrometer to the number entering that region" [178], has been mainly restricted by the loss of ions at the MS inlet and skimmer [186].

MS/MS

Tandem mass spectrometry (MS/MS) is a general term used for MS methods, where the sample ion ("precursor ion") of the m/z ratio of interest is selected and dissociated to generate fragment ions ("product ions"). Two main categories of instruments allow MS/MS analysis. One option is represented by two mass analyzers assembled in tandem, such as quadrupole time-of-flight q-TOF or triple quadrupole QqQ (tandem mass spectrometry in space). The second category includes mass analyzers able to store the ions, such as the ion cyclotron resonance (ICR) or the quadrupole ion trap (tandem mass spectrometry in time) [167, 187]. Different fragmentation techniques are available for ion fragmentation. In this regard, collision-induced dissociation (CID) is one of the most commonly applied method for ion fragmentation, wherein the molecular ions are moved to a region of higher potential that determines their acceleration and the increase in kinetic energy. The subsequent collision with neutral molecules, usually helium,

nitrogen or argon, yields the enhancement of the ions' internal energy. This results further in bond breakage and the fragmentation of the molecular ion into smaller fragments [188].

LC-MS/MS quantification (MRM)

Particularly when analyzing multiple components mixtures, liquid chromatography (LC) is usually coupled to ESI-MS with the aim to achieve improved sensitivity and specificity yielded by the chromatographic separation of the sample constituents. In contrast to MALDI-MS, LC-ESI-MS is commonly used for both qualitative and quantitative analysis of biological samples. In this respect, for structural analysis, LC is coupled to MS/MS instruments which offer high resolution ($R_{FWHM}=10^4$-10^6), such as q-TOF, Orbitrap or FTICR (Fourier-transform ion cyclotron resonance). Here, the mass resolution (R) is defined as $M/\Delta M$, where M designates the mass and ΔM the peak width necessary for separation at mass M. The resolution is given using the peak width definition by choosing the peak width measured at 50% of the maximum peak height, also known as Full Width of the peak at Half its Maximum height (FWHM) [178]. However, these instruments are generally less attractive for the quantification of biomolecules. Especially in the case of q-TOF devices, they provide high mass resolution but are restricted by a smaller linear dynamic range and lower sensitivity, compared to triple quadrupole instruments [189].

By contrast, for quantification purposes, LC coupled to triple quadrupole (QqQ) mass spectrometer represents the preferred instrumentation due to highly selective and sensitive quantitation capabilities. Nevertheless, a rather low resolution ($R_{FWHM}=10^3$) limits its qualitative applications. In tandem quadrupole MS, established in the late 1970s by Enke and Yost [190], the first and last mass analyzers (quadrupole 1 [Q1] and quadrupole 3 [Q3] respectively) are used as mass filters to select a precursor ion and its corresponding product ions. The second quadrupole (q2) is a non-mass-resolving, radio frequency (RF)-only quadrupole that acts as a cell for fragmentation (CID). The specific pair of m/z ratio values associated with the precursor and product ions is referred to as a "transition" and can be written as (precursor [m/z]) → (product [m/z]) [168]. This MS/MS approach is named selected or multiple reaction monitoring (SRM or MRM respectively).

In this context, the linear ion trap was also promoted as a suitable quantification tool. Here, the ability to apply a m/z ratio correlated resonant excitation frequency to the precursor ion, which is subsequently fragmented, typically affords a single-step fragmentation, since the product ions will have different mass-to charge ratios. This results is a product ion spectra with fewer ions at higher relative intensities. In contrast, the precursor ion accelerated through the pressurized collision cell (q2) in a triple quadrupole leads to a collision cascade or a multiple-step fragmentation, which tends to generate a large number of product ions and hence relatively lower abundance of the analyte ions. However, the MS/MS in ion trap offers a low duty cycle with a serial process occurring for collection, isolation of precursor ions, fragmentation and detection of product ions. This means that, especially when it comes to the analyte present in a low amount in the LC peak, there may not be sufficient time to fill the ion trap with enough ions to perform

an accurate quantitative measurement. On the contrary, the double-stage MRM analysis is carried out with a continuous ion beam affording a nearly 100% duty cycle which provides superior LOQ and improved reproducibility [191].

Hence, the triple quadrupole is the instrument of choice for quantification of biomolecules due to a high duty cycle and a large dynamic range when operated in MRM mode, granting superior analytical figures of merit such as sensitivity, precision and accuracy. Moreover, LC-MS/MS employing MRM transitions ensures high specificity due to a three-step separation of the analyte based on (1) retention time, (2) precursor m/z, and (3) fragment m/z. This can be particularly useful for the analysis of complex matrixes such as serum or urine [192].

Isotope dilution (IS)

The principle of LC-MS/MS quantification lies in the comparison of the signal intensity (MRM signal in the case of triple quadrupole instruments) of the analyte in an unknown sample to the signal intensity obtained in calibration standards of known concentration of the same analyte. However, the signal intensity in MS or MS/MS is not only dependent on the concentration of the analyte, but also on various instrumental parameters, which will not remain constant throughout the measurement. It is required to compensate for the instrumental variables by relative measurements of a so-called "internal standard" (IS). The principle of measurement by MS permits the use of a stable isotope-labeled analogue of the analyte as IS, in which case the technique is called "isotope dilution-mass spectrometry" (ID-MS). Isotope-labeled ISs have almost identical physicochemical properties to those of the analyte, thus they are superior to homologous or other structurally analogous ISs [193]. Nonetheless, mathematical treatment of the calibration data should be cautiously performed, especially in the case of peptide quantification, since the signal intensity of the monoisotopic mass of a stable isotope-labeled peptide differs from the unlabeled equivalent due to different isotope patterns. The magnitude of the variation (the so-called "response factor") depends on the elemental composition of the molecule, the number of labeled atoms, the type of labeled atom and the enrichment of the isotope-labeled starting material [194].

LC-MS quantification of hepcidin-25

Murphy *et al.* reported the first LC-MS/MS assay for quantification of hepcidin-25 in human and mouse serum employing Capcell Pak UG C18 column for sample fractionation and a triple quadrupole for MS/MS detection. Good intra- and inter-assay precision and accuracy were achieved (<20%). The LOD was reported at 1 µg/L. However, calcitonin gene-related peptide was used as IS, which is not ideal due to its different size and charge [195]. In-house synthetized isotope-labeled hepcidin-25^{+9} ($^{13}C_8{}^{15}N$) was employed by Bansal *et al.* to quantify serum hepcidin-25 using ultra-high-pressure liquid chromatography (UHPLC) coupled to a linear ion trap. The authors reported that the use of a triple quadrupole for Hep-25 analysis results in poorer fragmentation compared to a linear ion trap. The study used WCX magnetic nanoparticles for peptide extraction with a medium recovery (75%). Also, the calibration standards used were prepared with charcoal stripped serum [196], which is not a suitable analyte-free matrix due to

poor similarities to human serum. Intra-assay precision was reported <15% and a LOQ of 1.1 µg/L was achieved. Later, Itkonen *et al.* used a linear ion trap for Hep-25 quantification with a reported LOQ of 0.8 µg/L. The authors compared their MS-based method with a commercially available immunoassay and found no significant correlation [136]. Bros *et al.* employed LC coupled to high resolution (HR) MS using ion trap-Orbitrap for identification of impurities in hepcidin-25 standards. The instrumentation used proved to be adequate for qualitative analysis and revealed valuable information about the purity of commercially available standards. Methionine-oxidized and truncated N- and C-terminus forms were identified as impurities, underlining the need for a certified reference material [87]. However, the quantification of hepcidin-25 in biological samples employing HR-MS was not found suitable. As a consequence, the same group reported a quantification assay based on LC-MS/MS analysis using a triple quadrupole [197]. This assay was adapted from a quantification method developed previously by Delaby *et al.* for serum hepcidin-25, with a reported LOQ of 6 µg/L [198]. The latter study however did not include matrix effect and recovery determinations. LC-MS/MS using triple quadrupole was also employed by Wolff *et al.*, using isotope-labeled hepcidin-25 as IS. Oasis hydrophilic-lipophilic balanced reversed-phase (HLB) cartridges were used for sample preparation. The reported LOQ was 1.3 µg/L. However, no recovery data and matrix effects were reported [199].

To conclude, LC-MS/MS was used for the quantification of hepcidin-25, with triple quadrupole as the preferred mass analyzers. However, the use of inadequate analyte-free matrixes and the insufficient validation question the scientific value of these methods. It would be desirable for all the methods to be validated in compliance with the guidelines for clinical laboratory approved by CLSI (Clinical and Laboratory Standards Institute) [168]. These guidelines encompass verification procedures of validation parameters, namely linearity, specificity, precision and accuracy, specified by the International Conference on Harmonization (ICH) of analytical procedures [200]. Additionally, CLSI directives refer to the parameters specific for clinical samples, such as recovery and matrix effects.

Moreover, many difficulties in the analysis of hepcidin-25 were reported, such as the oxidation of the methionine residue, aggregation of hepcidin-25 in solution or adsorption of the peptide [87, 85, 160]. In this vein, the stability of hepcidin-25 standards in polypropylene and deactivated glass vials was tested. No significant peptide losses were reported [87]. However, the concentrations tested seemed to be in the order of mg/L, which is not relevant for the quantification range of 0.5-40 µg/L corresponding to the physiological levels of the peptide (see *section 1.3.3.3.*). Also, the influence of the solvent on Hep-25 concentrations was not tested.

One of the most common analytical challenges in LC-MS assays is the non-specific adsorption of peptides or proteins on solid surfaces, particles or matrix compounds. MS analysis can be hampered by the amphipathic character of the analyte, which makes it readily stick to plastic or glass surfaces, leading to adsorptive losses that can affect the accuracy of the measurement

results [201, 202]. In this regard, further improvement is necessary in the LC-MS/MS development and validation of quantification methods for hepcidin-25 to assure the transfer of these methodologies from research to clinical application.

1.3.3. Harmonization of hepcidin-25 measurements

1.3.3.1. International Hepcidin Harmonization Studies

Various MS-based methods and immunoassays have been developed for the measurement of hepcidin-25 concentration in biological samples. However, the reported serum hepcidin levels varied substantially between measurement procedures due to differences in methodology and analytical performance. Two international interlaboratory comparisons (the so-called Round Robin RR1 and RR2), organized by Radboud University Nijmegen, The Netherlands, addressed the method harmonization in hepcidin-25 analysis. In this respect, RR1 was organized in 2009 and included eight laboratories that analyzed identical urine and serum samples. Major differences between absolute hepcidin levels were reported [203]. Considering this, a second RR took place three years later, involving 21 participants. Similarly, identical serum samples were tested. Additionally, two commercially available synthetic hepcidin-25 standards were analyzed to assess their suitability as a material for harmonization. The various methods used for the analysis of the human samples (10 MS-based methods and 11 immunoassays) showed up to 10-fold differences between results [204].

These discrepancies are caused partly by the usage of different calibrators and the lack of a reference material, and partly because of the reason that the used immunoassays quantify all hepcidin isoforms together (Hep-20, -22, -24, -25) (see *section 1.3.1.*), while MS-based methods measure selectively the bioactive Hep-25. Other reports about hepcidin quantification compared “in house” developed LC-MS methods to commercially available immunoassays as reference method, with insufficient correlation [198, 136], most likely due to different analytes compared. Additionally, hepcidin was reported to bind to α-macroglobulin and albumin, but the found bound hepcidin fraction varied from 3% to 89% [205, 206]. It is unclear if the binding of Hep-25 to plasma proteins has any effect on the performance of the quantification assay or even its biological activity [76]. Also, it was reported that hepcidin-25 is a “sticky” peptide that can adhere to hydrophobic surfaces, a challenge that can be difficult to overcome in the MS analysis and can affect the accuracy of the results [84, 93, 55]. Methods recommended by the International Consortium for Harmonization of Clinical Laboratory Results were applied to further investigate interlaboratory variation of hepcidin-25 measurements. This resulted in the identification and production of a secondary reference material in the frame of the hepcidinanalysis.com initiative (conducted by the Department of Laboratory Medicine of the Radboud University Medical Center Nijmegen) [162, 132]. This is currently used as a calibrator set in an ongoing round robin study (Hepcidin Harmonization Study HHS2017) involving 10 laboratories worldwide (see *section 4.4.2.*) [manuscript in preparation]. Nevertheless, the assignment of

absolute Hep-25 concentration is hindered by the lack of a primary reference material or a certified reference material (CRM). LNE Paris (Laboratoire national de métrologie et d'essais) seem to have produced a primary reference material that is not commercially available so far [162].

1.3.3.2. Quantification methods for clinical use

The focus of harmonization in laboratory medicine is not only to harmonize and standardize the analytical methods, but also to include all other aspects of the total testing process (TTP), such as terminology and units, report formats, reference intervals and decision limits, as well as tests and test profiles request and criteria for interpretation [181]. In this vein, some quantification methods for Hep-25 are undergoing clinical approval (Table 1.5). The relevance of hepcidin-25 as a clinical parameter is supported by an increasing number of clinical studies showing positive diagnostic performance of serum hepcidin in different clinical settings [76].

Table 1.5: Hepcidin assays available for clinical use [76].

	Method	Institution / Company	Clinical credentials	Ref.
MS methods	WCX-TOF-MS	Radboud University, hepcidinanalysis.com, Nijmegen (NL)	Patient care: included in scope of accreditation of clinical laboratory (Dutch CCKL/RvA Code of Practice, in transation to EN ISO 15189).	[93]
	LC-MS/MS (reversed phase extraction)	University Hospital of Verona (IT)	Embedded in laboratory accredited by Regional Health authority (Veneto Region law no. 838, April 8, 2008).	[207]
	LC-MS/MS (Hydrophilic-lipophilic-balanced (HLB) extraction)	HUSLAB at Helsinki University Central Hospital (FI)	Embedded in accredited clinical laboratory (Finnish Accreditation Service, T055, EN ISO/IEC 17025, EN ISO 15189)	[136]
Immunoassays	Sandwich ELISA	Eli Lilly and Company (US)	Clinical trials: CRO partner has validated test in GLP-like manner.	[144]
	Competitive ELISA	DRG Instruments (DE)	CE-marked, approved as in vitro medical device (IVD directive 98/87/EC)	[208]
	Competitive ELISA	Intrinsic LifeSciences, La Jolla (US)	Compliant with Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologist (CAP) standards	[141]

In this context, MS-based methods seem to have a more advanced status over immunoassays in terms of hepcidin-25 quantification in clinical environment, probably also due to the lack of suitable antibodies against the bioactive isoform (see *section 1.3.1.*). Furthermore, isotope dilution (IS) LC-MS/MS is widely recognized as the "gold standard" for quantification of biomolecules by accredited bodies such as Clinical and Laboratory Standards Institute (CLSI) [168] or National Institute of Standards and Technology (NIST) [5]. Nevertheless, further efforts towards increasing the availability of validated assays followed by their clinical implementation are vital for universal recognition of this promising biomarker [76].

1.3.3.3. Physiological levels of Hep-25

The assignment of universal reference ranges for hepcidin-25 is hampered by the considerable variations in the physiological serum levels reported in humans. On one hand, the "intra-method" Hep-25 levels vary widely in healthy subjects, which are reflected in large physiological reference ranges. A study of 100 healthy patients reported a concentration range of <0.02 – 25 µg/L, with 44% of the population showing levels < 0.9 µg/L [144]. Moreover, studies revealing diurnal fluctuations of Hep-25 concentrations suggest that the moment of the day when the serum is collected represents an influencing factor in hepcidin-25 quantification [209, 210]. Additionally, a day-to-day variation of hepcidin-25 concentration was also reported. It was shown that within an individual, the peptide hormone level could vary as much as 2- to 5-fold from day to day [209, 195]. On the other hand, the "inter-method" hepcidin-25 concentrations vary substantially, as presented in *section 1.3.3.1.* The comparison between different values reported is even more difficult because some methods report the mean value of hepcidin-25, either as arithmetic mean (average) or as geometric mean, whereas other methods list the median Hep-25 value.

It must be mentioned that the hepcidinanalysis.com initiative reports reference ranges based on a population of almost 2000 healthy individuals. However, the values of the concentrations are ambiguous since these do not represent direct analytical measurements, rather calculated values based on a mathematical formula involving 2 methodologies, a WCX-MALDI-TOF method for Hep-25 quantification and an ELISA that measures total hepcidin [162]. Other noteworthy reports suggested reference ranges for serum hepcidin-25 (Table 1.6).

Taking into consideration the values reported by the research group of Eli Lilly and Company (Indianapolis, IN, USA), which applied both an immunoassay selective for Hep-25 and a LC-MS/MS method for the quantification of the iron-regulator, the present work defines the median physiological level of Hep-25 in humans between 1-5 µg/L and the mean level (arithmetic mean or average) between 5-15 µg/L. In this respect, it has been reported that several samples are found to be <LOQ, which indicates that the median value could be more relevant than the average value. However, it is desirable that both values are reported, together with the level range determined, since hepcidin-25 serum concentrations seem to vary widely.

Table 1.6: Physiological levels of hepcidin-25 in humans reported by selected methodologies.

Institution/ Company	Quantification method	Patient no.	Mean (µg/L)	Median (µg/L)	Concentration range (µg/L)	Ref.
Elli Lilly and Company (US)	Sandwich ELISA	100	N/A	1.2	<0.02 - 25	[144]
Elli Lilly and Company (US)	LC-MS/MS	10	9	3.6	<1.0 - 45.6	[195]
HUSLAB/ University of Helsinki (FI)	LC-MS/MS	Men: 109 Women: 66	Men: 14 Women: 7	Men: 12 Women: 5.9	Men: 3.1 – 43.5 Women: 1.1 – 25.7	[136]
hepcidinanalysis.com (NL)	WCX-TOF-MS*	Men: 1066 Women: 882	N/A	Men: 12.6 Women: 10	Men: <1.4 – 41 Women: <1.4 – 40.7	[162]

* *The reference levels for the WCX-TOF MS method are recalculated from those of an ELISA method based on the regression line: (ELISA - 1.00)/1.52 = WCX-TOF MS [162].*

2. Materials and Methods

2.1. Chemicals, reagents and biological samples

The chemical substances used during this study were of highest grade of purity available. In this regard, Table 2.1 summarizes solids and liquids, as well as peptides and mass calibration solutions used throughout this work, together with their corresponding grade of purity, supplier and product number.

Table 2.1: List of chemicals used during this work.

Chemical	Purity	Supplier	Product number
Solids and liquids			
1H,1H,2H,2H-Perfluoro-octyltriethoxysilane	98%	Sigma-Aldrich, Taufkirchen, DE	667420
3-(2-Aminoethylamino) propylmethyl-dimethoxysilane	97%	Alfa Aesar, Karlsruhe, DE	B23159
(3-Aminopropyl) trimethoxysilane	97%	Sigma-Aldrich, Taufkirchen, DE	281778
Acetic acid glacial	HPLC grade	AppliChem, Darmstadt, DE	361008.161
Acetonitrile	LC-MS grade	ChemSolute, Renningen, DE	2697
Ammonia solution 20%	LC-MS grade	Carl Roth, Karlsruhe, DE	hN66.2
Ammonium acetate	>99.9995%	Sigma-Aldrich, Taufkirchen, DE	101707138
Ammonium hydroxide solution 28.0-30.0%	ACS reagent	Sigma-Aldrich, Taufkirchen, DE	221228-M
Buffer solution pH 4.01	N/A	Hanna Instruments, DE	HI7004
Buffer solution pH 7.01	N/A	Hanna Instruments, DE	HI7007
Buffer solution pH 10.01	N/A	Hanna Instruments, DE	HI7010
Citric acid	BioXtra (≥99.5%)	Sigma-Aldrich, Taufkirchen, DE	C0706
Copper sulfate solution 0.1 M	Titripur®	Sigma-Aldrich, Taufkirchen, DE	35185
Cysteine	≥98%	Sigma-Aldrich, Taufkirchen, DE	C7352
L-Cystine	≥98%	Sigma-Aldrich, Taufkirchen, DE	C8755
DY-654-NHS	N/A	Dyomics, Jena, DE	654-01
EDTA	BioUltra (≥99%)	Sigma-Aldrich, Taufkirchen, DE	EDS
Formic acid	LC-MS grade	Sigma-Aldrich, Taufkirchen, DE	5.33002
Glutathione (oxidized)	>98%	Sigma-Aldrich, Taufkirchen, DE	G4376
Glutathione (reduced)	>98%	Thermo Fischer, Darmstadt, DE	BP2521-10
Guanidine hydrochloride	≥99 %	Sigma-Aldrich, Taufkirchen, DE	G3272
HEPES	≥99,5 %	Sigma-Aldrich, Taufkirchen, DE	H4034
Hydrochloric acid ≥32%	BioUltra	Sigma-Aldrich, Taufkirchen, DE	84415
Iso-propanol	LC-MS grade	ChemSolute, Renningen, DE	1164
Methanol	HPLC grade	ChemSolute, Renningen, DE	1481
Mucasol	N/A.	Schülke&Mayr, Norderstedt, DE	230191
Ortho-phosphoric acid 85%	HPLC grade	Honeywell, USA	79606

Potassium phosphate monobasic	BioUltra (>99.5%)	Sigma-Aldrich, Taufkirchen, DE	60218
Sodium bicarbonate	99.5%	AppliChem, Darmstadt, DE	A1940.1000
Sodium chloride	BioUltra (>99.5%)	Sigma-Aldrich, Taufkirchen, DE	71376
Sodium citrate tribasic dihydrate	BioUltra (>99.5%)	Sigma-Aldrich, Taufkirchen, DE	71402
Sodium hydroxide	ACS reagent (>98%)	Sigma-Aldrich, Taufkirchen, DE	30620-M
Sodium phosphate monobasic dihydrate	BioUltra (≥99%)	Sigma-Aldrich, Taufkirchen, DE	71643
Sodium phosphate dibasic dihydrate	BioXtra (≥99%)	Sigma-Aldrich, Taufkirchen, DE	S7907
Sodium tetraborate decahydrate	BioXtra (≥99.5%)	Sigma-Aldrich, Taufkirchen, DE	B3545
Toluene	≥99,5 %	Carl Roth, Karlsruhe, DE	9558.3
Trifluoroacetic acid	LC-MS grade	Honeywell, US	14264
α-Cyano-4-hydroxycinnamic acid	N/A	Bruker-Daltonik, Bremen, DE	8255344
Peptides			
Human hepcidin-25 (linear)	>95%	peptides&elephants, Hennigsdorf, DE	1006P06
Human hepcidin-25 (folded)	>95% (HPLC)	Bachem, Bubendorf, CH	H5926.0500
[$^{13}C_9$,^{15}N]Phe^{4,9}, [^{15}N]Gly^{12}-Hepcidin-25	>99% (HPLC)	Peptide Institute, Osaka, JP	3405-v
Peptide DTHFPI-NH_2	99.9%	peptides&elephants, Hennigsdorf, DE	lot 1211N01
Mass calibration solutions			
MS Chemical Kit 1, Low-High Conc. PPGs	N/A	AB Sciex, Darmstadt, DE	4406127
Peptide Calibration Standard II	N/A	Bruker-Daltonik, Bremen, DE	8222570
Pierce LTQ ESI Positive Ion Calibration solution	N/A	Thermo Fischer, Darmstadt, DE	88322

N/A = not available

Purified laboratory water was obtained from a Milli-Q water-purification system (Millipore, Schwalbach, Germany). Serum samples used within the present work were obtained from various sources. Sterile-filtered sheep serum and bovine serum were purchased from Sigma-Aldrich, Taufkirchen, Germany (S2263 and B9433 respectively). Human serum samples were obtained from Dunn Labortechnik, Asbach, Germany. These human sera were collected from healthy volunteers at an FDA licensed commercial donor center/facility within the Unites States and were found negative for: HBsAg, HCV, HIV-1, HIV-2, HIV-1Ag or HIV-1-NAT, ALT and syphilis. Human serum samples for the experiments within the Hepcidin Harmonization Study (HHS2017) conducted by Radboud University Nijmegen and hepcidinanalysis.com were delivered from Radboud University Medical

Center, Nijmegen, The Netherlands, where the sample collection was conducted in accordance with the local Ethics committee. Particular care was addressed to the laboratory ware used during this work. Low binding reaction tubes (LoBind Eppendorf tubes) and standard Eppendorf tubes were purchased from Eppendorf, Wesseling-Berzdorf, Germany. HPLC glass vials used for silanization (see *section 2.3.2.*) were obtained from Wicom, Heppenheim, Germany. Low adsorption (LA) glass vials were purchased from Supelco, Bellefonte, PA, USA, and polypropylene (PP) vials from Thermo Fischer, Darmstadt, Germany.

2.2. Synthesis of conjugates and metal complexes

2.2.1. Buffers

Table 2.2 presents the buffers employed in this study for folding linear hepcidin-25, labeling folded Hep-25, preparation of peptide-metal complexes and solubility tests.
The pH adjustments were performed using a pH meter Hanna 211 (Hanna Instruments, Vöhringen, Germany), calibrated daily using buffer solutions at pH 4.01, 7.01 and 10.01.

Table 2.2: List of buffers used during this work.

Buffer	Recipe
Folding buffer	0.4 mM reduced glutathione (GSH) 0.4 mM oxidized glutathione (GSSG) [85] pH 7 (adjusted with ammonium hydroxide solution 30%)
Borate buffer	50 mM sodium tetraborate decahydrate ($Na_2B_4O_7 \cdot 10\ H_2O$) pH 8 (adjusted with sodium hydroxide 20% and hydrochloric acid 20%)
Ammonium acetate buffer	10 mM ammonium acetate (CH_3COONH_4) (a) pH=8.3 (adjusted with ammonia solution 20%) (b) pH=7.4 (adjusted with ammonia solution 20%)
Sodium bicarbonate buffer	0.1 M sodium bicarbonate ($NaHCO_3$) pH 7.8/8.5 (adjusted with sodium hydroxide 20% and hydrochloric acid 20%)
Phosphate-buffered saline (PBS) buffer	10 mM sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2\ H_2O$) 2.3 mM potassium phosphate monobasic (KH_2PO_4) 137 mM sodium chloride (NaCl) pH 7.4
Citrate buffer	46 mM sodium citrate tribasic dihydrate ($Na_3C_6H_5O_7 \cdot 2\ H_2O$) 54 mM citric acid ($C_6H_8O_7$) pH 4.2 (adjusted with sodium hydroxide 20% and hydrochloric acid 20%)
Phosphate buffer	100mM sodium phosphate monobasic dihydrate ($NaH_2PO_4 \cdot 10\ H_2O$) 137 mM sodium chloride (NaCl) pH 2.2 (adjusted with phosphoric acid H_3PO_4)
HEPES buffer	(a) 0.1 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (b) 30 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH 7.4 (adjusted with sodium hydroxide 20% and hydrochloric acid 20%)

2.2.2. Peptide DTHFPI-copper(II) complex

The peptide DTHFPI-$CONH_2$ with a reported molecular weight of 729.6 was dissolved in 30 mM HEPES buffer (pH 7.4) to a concentration of 10 mM. The stock solution was immediately aliquoted into 100 µL fractions in LoBind Eppendorf tubes and stored at -20°C until application in the experimental process.
One aliquot (100 µL) of 10 mM peptide DTHFPI-$CONH_2$ in 30 mM HEPES at pH 7.4 was thawed right before use. The peptide solution was titrated with subsequent additions of 1 µL of 0.1 M $CuSO_4$ standard solution. The formation of the DTHFPI-Cu^{2+} complex was observed in a molar ratio peptide:Cu^{2+} ranging from 1:0.1 to 1:2. Each addition of the Cu^{2+} solution was followed by thorough vortexing and UV-Vis measurements (see *section 2.4.1.*).

2.2.3. Production of folded hepcidin-25

Lyophilized linear hepcidin-25 (DTHFPICIFCCGCCHRSKCGMCCKT-$CONH_2$) with a reported molecular weight of 2797.8 was re-suspended in distilled water to a concentration of 10 g/L. The stock solution was immediately aliquoted into 100 µL fractions in LoBind Eppendorf tubes and stored at -20°C until use. For each folding experiment, a freshly thawed aliquot (100 µL) containing 1 mg of linear hepcidin-25 was added to 30 mL folding solution consisting of acetonitrile:folding buffer (see *section 2.2.1.*) 20:80 (v/v). Linear hepcidin-25 was oxidized overnight (16-24 h) under stirring at 100 rpm using a magnetic stirrer (Carl Roth, Karlsruhe, Germany). Then, the solution was acidified by addition of TFA (0.1%) [211] and further loaded onto a Zorbax C18 LC column (4.6x150mm, 5µm) for preparative purification, using a HPLC pump (Gynkotek, model 480) at a flow rate of 0.5 mL/min. Chromatographic set 1 was employed for purification (see *section 2.4.2.2.*). Fractions between minute 14 and minute 16 containing folded hepcidin were collected manually and subsequent HPLC analysis of the fractions was performed (Chromatographic set 3). The fractions containing folded hepcidin were stored in the separation mobile phase (water/acetonitrile/TFA approximately 60/40/0.1 v/v/v) at 4°C until further use. MALDI-MS was employed for exact mass confirmation. To assess the yield of the folding process, HPLC quantification of hepcidin was achieved using chromatographic set 3. Seven standards were prepared in the dynamic range 6.25 – 250 mg/L (6.25, 12.5, 25, 50, 100, 150, 250 mg/L) using water/acetonitrile/TFA 60/40/0.1 v/v/v as dilution solvent. The quantification of folded hepcidin-25 will be discussed in detail in *section 2.5.3.*

2.2.4. Synthesis of hepcidin-25 labeled with a fluorescent tag

One aliquot (100 µL) containing 0.1 mg of human hepcidin-25 (see *section 2.3.1.1.*), thawed immediately before use, was introduced in a vacuum oven (VDL 23, Binder, Tut-

tlingen, Germany) set at room temperature until dryness. This dried residue was then dissolved in 200 µL reaction buffer consisting of a mixture of 0.1 M $NaHCO_3$ (pH 8.5)/methanol 50/50 (v/v). The fluorescent dye solution was prepared by dissolving DY-654-NHS in the reaction buffer at a concentration of 2 g/L immediately before use. 30 µL of dye solution was added to the peptide solution for a molar ratio peptide:dye of 1:1.5. The mixture was incubated overnight at room temperature, in the dark, and further purified by HPLC (Azura UHPLC). Chromatographic separation was achieved using chromatographic set 2 (see *section 3.4.2.2.*).

2.2.5. Solubility tests

Hepcidin-25 folded "in house", stored in water/acetonitrile/TFA approximately 60/40/0.1 v/v/v (see *section 3.2.2.*), was used. Aliquots of 50 µL hepcidin solution 130 mg/L were introduced in a vacuum oven (VDL 23, Binder, Tuttlingen, Germany) set at room temperature until dryness. Different solvent mixtures and buffers were used as reconstitution solvents (50 µL) as follows: water/acetonitrile/TFA 60/40/0.1 (v/v/v), water, 20% methanol (v/v), 50% methanol (v/v), 100% methanol, 1% acetic acid (v/v), 20% acetic acid (v/v), 100% acetic acid, acetic acid/methanol 1%/20% (v/v), 50mM borate buffer (pH 8), 50mM borate (pH 8)/methanol 80/20 (v/v), 10 mM ammonium acetate (pH 8.3), 10 mM ammonium acetate (pH 8.3)/methanol 80/20 (v/v), 0.1 M sodium bicarbonate buffer (pH 7.8), 0.1 M sodium bicarbonate buffer (pH 7.8)/methanol 80/20 (v/v), PBS buffer (pH 7.4) and PBS buffer (pH 7.4)/methanol 50/50 (v/v) (see *section 2.1.* and *section 2.2.1.*). Chromatographic set 1 (see *section 2.4.2.2.*) was used for HPLC analysis of the peptide solutions. 10 µL were injected in duplicates.

2.2.6. Hepcidin-25-copper(II) complex

2.2.6.1. LC-ESI-QqQ

A freshly thawed aliquot of 50 µL of human hepcidin-25 (1 g/L) (see *section 2.3.1.1.*) was used. Fractions of 7.5 µL of hepcidin-25 (7.5 µg) were pipetted in an amino-silanized vial and incubated in the vacuum oven (VDL 23, Binder, Tuttlingen, Germany), set at room temperature, until dryness. A 0.1 mM Cu^{2+} solution was prepared by diluting an aqueous 0.1 M $CuSO_4$ solution using the solvent mixture at the start of the HPLC gradient: mobile phase A (pH 11)/mobile phase 95/5 (v/v) B (chromatographic set 4, see *section 2.4.2.2.*). The synthesis of the hepcidin-25-copper(II) complex was carried out at pH 11 by adding the $CuSO_4$ solution (final concentration 1.8-180 µM) to the hepcidin-25 residue (final concentration 18 µM) in a 0.1 to 10-fold molar excess according to Table 2.3. This resulted in a hepcidin-copper(II) complex concentration of 50 mg/L (18 µM) for a total volume of 150 µL. For a molar ratio hepcidin-25:Cu^{2+} of 1:10, 1 µL of 10 mM $CuSO_4$ (pH 11) was added

to the prepared solution containing hepcidin-25-copper(II) 1:5 (molar ratio). The pH was controlled at every step using universal indicator paper (pH range 1-14).

Table 2.3: Synthesis of hepcidin-25-Cu(II) complex in different molar ratios.

Molar ratio Hepcidin-25:Cu^{2+}	Volume (µL) 0.1 mM $CuSO_4$ (pH 11)	Volume (µL) H_20/ACN/NH_3 (pH 11)*	Conc. hepcidin-25 in final solution (µM)	Conc. Cu^{2+} in final solution (µM)
Copper free	0	150	18	0
1:0.1	2.7	147.3	18	1.8
1:1	27	123	18	18
1:5	135	15	18	90

* mobile phase A (pH 11)/mobile phase B (chromatographic set 4) 95/5 (v/v)

For the experiments at different pH values, hepcidin-25 folded in house was used (see *section 2.2.3.*). 150 µL of $CuSO_4$ in different buffer solutions at a final concentration of 18 µM was added in a molar ratio of approximately 1:1 to dried "in house" folded hepcidin-25 for a concentration of the peptide-metal complex of 50 mg/L (0.018 mM). For this purpose, 50 µL solution of hepcidin-25 folded "in house" (150 mg/L) was dried out by using a vacuum oven set at room temperature. 100 mM phosphate buffer (pH 2.2), 100 mM citrate buffer (pH 4.2) and 50 mM borate buffer (pH 7.4) (see *section 3.2.1.*) were used for the preparation of peptide-copper(II) solutions.

2.2.6.2. ESI-FTICR

Hepcidin-copper(II) species were analyzed by applying direct infusion of the sample. For this purpose, volatile solvents/buffers were employed. A mixture of water/ACN/NH_3 95/5/0.1 v/v/v (pH 11), and 10 mM ammonium acetate (pH 7.4) were used in an identical protocol as described in *section 2.2.6.1* for obtaining copper(II) complexes at molar ratio of 1:1 and 1:10.

2.3. Sample preparation

2.3.1. Preparation of serum samples for hepcidin-25 quantification

The serum samples (human/sheep serum) were stored at -20°C (not more than 6 months) and equilibrated at room temperature before use. These were not kept more than 1 hour at room temperature after thawing. The sera were spiked with "heavy" hepcidin standards (human/sheep serum) and "light" hepcidin standards (sheep serum), followed by different sample cleaning methods to remove the high molecular weight compounds such as proteins. An exception from this protocol was made for the recovery tests (see *section 3.5.2.*).

2.3.1.1. Preparation of standards

The stock solutions of "light" and "heavy" human hepcidin-25 were prepared based on the nominal value of peptide reported by the supplier. The lyophilized peptide was directly resuspended in the solvent indicated by the manufacturer (see below). Further dilutions were prepared using a mixture of water/ACN/TFA 60/38/2 v/v/v as dilution solvent. All the stock solutions were prepared gravimetrically using a XS105DU balance (Mettler Toledo, Gießen, Germany). In this regard, the densities were considered according to DDB (Dortmund Data Bank) as 0.998 for water and 1% acetic acid, 0.783 for acetonitrile and 0.903 for the mixture water/ACN/TFA [212]. Serial dilutions to create a linearity set were avoided. Lyophilized human hepcidin-25, also referred to as "light" hepcidin-25 in LC-MS experiments, was characterized by a reported molecular weight of 2789.12 (chemical formula $C_{113}H_{170}N_{34}O_{31}S_9$) and a disulfide connectivity between Cys7-Cys23, Cys10-Cys13, Cys11-Cys19 and Cys14-Cys22 (as stated by the manufacturer). The lyophilized peptide (0.5 mg) was resuspended in 1% acetic acid to a concentration of 1 g/L, according to the manufacturer's instructions. The stock solution was immediately aliquoted into 50 µL or 100 µL fractions in amino-silanized autosampler vials and stored at -20°C until being used in experiments. Lyophilized ^{13}C,^{15}N isotope-labeled hepcidin-25 ([$^{13}C_9$,^{15}N]Phe^{4,9}, [^{15}N]Gly^{12}-hepcidin-25), also referred to as "heavy" hepcidin-25, was characterized by a reported molecular weight of 2810.20 (chemical formula $C_{95}{}^{13}C_{18}H_{170}N_{31}{}^{15}N_3O_{31}S_9$) and a disulfide connectivity between Cys7-Cys23, Cys10-Cys13, Cys11-Cys19 and Cys14-Cys22 (as stated by the manufacturer). The lyophilized peptide (24 µg) was resuspended in distilled water to a concentration of 56 mg/L, according to the manufacturer's instructions. The stock solution was immediately aliquoted into 50 µL fractions in amino-silanized autosampler vials and stored at -20°C until use.

2.3.1.2. Protocol I

Protocol I of serum sample preparation included a step of protein precipitation for the depletion of serum proteins using an organic solvent (acetonitrile ACN) and an acid (trifluoroacetic acid TFA). 300 µL of serum sample (human and sheep serum) were transferred to a 1.5 mL low binding plastic tube (Eppendorf, Wesseling-Berzdorf, Germany). Further, 20 µL isotope enriched "heavy" hepcidin-25 standard 250 µg/L was added for a final concentration of 10 µg/L and mixed thoroughly by using a vortex mixer. The sheep serum samples were subsequently spiked with appropriate dilutions of hepcidin-25 stock solution to prepare calibration standards in a concentration range of 0.5-40 µg/L (0.5, 1, 2, 5, 10, 20, 40) and vortexed thoroughly. The total sample volume was adjusted to 500 µL by the addition of a mixture of ACN/TFA 95/5 v/v to the spiked serum, while the sample was continuously vortexed, to give a final concentration of approximately 38% ACN and 2% TFA. The samples were incubated for 10 minutes at room temperature. Subsequently, the precipitate was

separated by centrifugation (21500 RCF, 10 min, 4°C) using a Mikro 220R centrifuge (Hettich, Bäch, Switzerland) with a fixed angle rotor. Further, the supernatant was transferred to a fluoro-silanized glass vial, followed by LC-MS/MS analysis using TFA-based mobile phases (chromatographic set 3).

2.3.1.3. Protocol II

Protocol II of serum sample preparation included a step of protein precipitation using an organic solvent (acetonitrile ACN) and an acid (trifluoroacetic acid TFA), similarly as in Protocol I, followed by an additional clean-up step of ultrafiltration using centrifugal filters and a pre-concentration step employing freeze-drying (lyophilization). 1.2 mL of serum sample (human and sheep serum) were added to a 2 mL low binding plastic tube (Eppendorf, Wesseling-Berzdorf, Germany), further mixed with 20 µL isotope enriched "heavy" hepcidin-25 standard 1 mg/L to a final concentration of 10 µg/L and vortexed thoroughly. Similarly as in Protocol I, the sheep serum samples were subsequently spiked with appropriate dilutions of hepcidin-25 stock solution to prepare calibration standards in a concentration range of 0.3-40 µg/L (0.3, 0.5, 1, 2, 5, 10, 20, 40) and mixed thoroughly using a vortex mixer. Then, a mixture of ACN/TFA 95/5 v/v was added to the spiked serum sample and the total volume was adjusted to 2000 µL, to give a concentration of approximately 38% ACN and 2% TFA. The samples were incubated for 10 minutes at room temperature, followed by separation of the precipitate using centrifugation (21500 RCF, 20 min, 4°C) with a fixed angle rotor. A Mikro 220R centrifuge (Hettich, Bäch, Switzerland) was applied for this purpose. 1 mL of supernatant was transferred to a centrifugal filter Vivaspin 2, 5 kDa, Hydrosart (Sartorius, Göttingen, Germany, VS02H11). The samples were centrifuged at 3300 RCF for 220 min at 4°C using an Eppendorf 6600 centrifuge (Eppendorf, Hamburg, Germany) with a swinging-bucket rotor. 450 µL of the obtained filtrate was transferred to a 1.5 mL low binding plastic tube and immersed in liquid nitrogen in preparation for freeze-drying. The lyophilization was carried out overnight using a freeze dryer Lyovac GT2. The residue was resuspended in 120 µL water/ACN/TFA 60/38/2 v/v/v and transferred to a fluoro-silanized glass vial for LC-MS/MS analysis using NH_3-based mobile phases (chromatographic set 4).

2.3.1.4. International Hepcidin Harmonization Study (HHS2017)

Human serum samples (low, medium and high level in duplicates) and lyophilized calibrator set (low and medium level) were shipped on dry ice (-80°C) from Nijmegen, The Netherlands, in the frame of the Hepcidin Harmonization Study (HHS2017).
The human samples (300 µL each) were stored at -80°C until use. These were not left at room temperature any longer than 4 hours after thawing, according to received instructions, and were prepared according to Protocol I. Shortly, 20 µL isotope enriched "heavy" hepcidin-25 standard 250 µg/L was added to 300 µL of serum sample for a final concentration

of 10 µg/L and mixed thoroughly by using a vortex mixer. A mixture of ACN/TFA 95/5 v/v was added further, while the sample was continuously vortexed, up to a total volume of 500 µL, to give a final concentration of approximately 38% ACN and 2% TFA. The sample preparation for hepcidin-25 extraction was performed within the company Proteome Factory, Berlin.
The lyophilized calibrator set (Calibrator low: 2016.1461, Calibrator middle: 2016.1462, lot 2016-146X) was kept at a temperature of 4°C after arrival, following the received instructions. Before its use for calibration, 0.3 mL of distilled water was added according to manufacturer's instructions. The calibrator set was prepared gravimetrically by considering the density of water 0.998. The vial was kept upright at room temperature for 15 minutes, and then carefully mixed for 20 minutes, according to the manufacturer's instructions. Further, the product was processed as patient material employing Protocol I.

2.3.2. Preparation of silanized glass vials

Standard glass autosampler vials (Wicom, Heppenheim, Germany) were used for silanization. Three types of silanes, 1H,1H,2H,2H-perfluorooctyltriethoxysilane, 3-(2-aminoethylamino)-propylmethyl-dimethoxysilane and (3-aminopropyl)trimethoxysilane, were used for the preparation of fluoro- and amino-silanized vials respectively. The vials were rinsed with a solution of Mucasol 2%, followed by an activation step with a solution of HCl 10% (30 min). Further, several cycles of isopropanol and toluene rinsing were performed. 1% silane solution (v/v) was prepared in toluene containing 1% of distilled water (v/v). The vials were incubated overnight at room temperature in the silane solution. Then, the silane solution was discarded, followed by serial rinsing of the vials with toluene, isopropanol and acetonitrile. The vials were left to dry overnight under the hood and kept at room temperature until use.

2.4. Instrumentation

2.4.1. UV-Vis spectroscopy

UV-Vis measurements were performed using an Evolution 220 UV-Visible spectrophotometer (Thermo Fischer Scientific, Dreieich, Germany) with a Double Beam (sample and reference cuvette positions), Czerny-Turner Monochromator, Xenon Flash Lamp, Dual Silicon Photodiodes (detector) and a wavelength rage of 100 to 1100 nm. Spectra were recorded in the range of 200 to 700 nm, using a wavelength width of 2 nm. Quartz cuvettes (Hellma, Müllheim, Germany) with a volume of 100 µL (minimum volume of 50 µL) were used.

2.4.2. Liquid chromatography (LC)

2.4.2.1. HPLC systems

For solubility tests and folding experiments, an Agilent 1260 Infinity Bio-inert LC system (Agilent Technologies, Waldbronn, Germany) with a quaternary pump, de-gasser, autosampler, thermostat, column heater and UV-detector (diode-array detector DAD) was used.

An Azura Analytical (U)HPLC (Knauer, Berlin, Germany) with a binary pump, de-gasser, autosampler, column heater, UV-detector, FL-detector (fluorescence detector) and fraction collector was employed for purification of labeled hepcidin-25.

For chromatographic experiments with the AB Sciex 6500, an Agilent 1260 Infinity LC system (Agilent Technologies, Waldbronn, Germany) with a binary pump, degasser, autosampler, column heater and UV-detector (diode-array detector, DAD) was coupled to the mass spectrometer.

2.4.2.2. HPLC columns and related parameters

Four chromatographic sets were used during this work, each characterized by a HPLC chromatographic column, mobile phases with specific gradient, a flow rate and an oven temperature.

Chromatographic set 1

Chromatographic separation was achieved on a Zorbax C18 column, 4.6x150 mm, 5 µm (Agilent Technologies, Waldbronn, Germany).

The mobile phases were (A) water with 0.1% TFA (v/v) and (B) ACN with 0.08% TFA (v/v). The flow rate used was 1 mL/min and the column oven temperature was set to 50°C. The elution gradient is presented in Table 2.4. The injection volume used was generally 5-10 µL.

Table 2.4: Gradient applied in chromatographic set 1.

Time (min)	Concentration B (%)
0	20
5	20
35	60
36	60
50	20

The instrumentation employed was Agilent 1260 Infinity Bio-inert.

Chromatographic set 2

Chromatographic separation was achieved on a Kinetex X_B-C18 column, 2.6x150 mm, 3 µm (Phenomenex, Aschaffenburg, Germany) with a UHPLC C18, 3 mm Phenomenex column guard.

The mobile phases were (A) water with 10 mM ammonium acetate and 0.1% acetic acid (v/v) and (B) methanol with 10mM ammonium acetate and 0.1% acetic acid (v/v). A flow rate of 350 µL/min was applied and the oven temperature was set to 50°C. The elution gradient is presented in Table 2.5. The injection volume was 27 µL (maximum injection volume allowed by the autosampler).

Table 2.5: Gradient applied in chromatographic set 2.

Time (min)	Concentration B (%)
0	20
3	20
8	95
12	95
14	20
22	20

Chromatographic set 2 was applied using Azura® UHPLC.

Chromatographic set 3

Chromatographic separation was achieved on an ACE UltraCore Super C18 column 2.1x50 mm, 5 µm, with an ACE Excel pre-column filter (ACE, Aberdeen, Scotland).

Table 2.6: Gradient applied in chromatographic set 3.

Time (min)	Concentration B (%)
0	5
2	5
4	95
7	95
7.5	5
14	5

The mobile phases were (A) 90% water/10% ACN (v/v) with 0.1% TFA (v/v) and (B) 10% water/90% ACN (v/v) with 0.1% TFA (v/v). The flow rate employed was 0.6 mL/min and the column heater temperature was set to 40°C. The elution gradient is presented in Table 2.6. Generally, the injection volume employed was 10 µL, unless specified otherwise (LC-MS/MS quantification of serum samples, adsorption experiments and other experiments).The instrumentation applied was Agilent 1260 Infinity coupled to AB Sciex 6500.

Chromatographic set 4

An ACE UltraCore SuperPhenylHexyl column 2.1x50 mm, 5 µm, with an ACE Excel pre-column filter (ACE, Aberdeen, Scotland) was used for chromatographic separation. ACE UltraCore columns (SuperC18 and SuperPhenylhexyl) feature the Encapsulated Bonding Technology (EBT^{TM}) that increases ligand coverage of the silica surface and eliminates the negative effects of un-bonded silanol groups. This offers excellent inertness and extended pH stability (recommended pH range 1.5-11.0).

Table 2.7: Gradient applied in chromatographic set 4.

Time (min)	Concentration B (%)
0	5
2	5
7	95
9	95
9.5	5
15	5

The mobile phases were (A) water with 0.1% NH_3 (v/v) and (B) 10% water/90% ACN (v/v) with 0.1% NH_3 (v/v). A flow rate of 0.6 mL/min was applied and the column oven temperature was set to 40°C. The elution gradient is presented in Table 2.7. The injection volume employed for LC-MS/MS quantification of serum samples was 30 µL. An injection volume of 10 µL was used for LC-MS analysis of the hepcidin-25-copper(II) complex(es) and other experiments.
The instrumentation used was Agilent 1260 Infinity coupled to AB Sciex 6500.

2.4.3. Electrospray ionization mass spectrometry (ESI-MS)

2.4.3.1. Triple Quadrupole (QqQ)

A triple quadrupole mass spectrometer Triple Quad™ AB Sciex 6500 (AB Sciex, Darmstadt, Germany) with electrospray ionization (ESI) IonDrive™ Turbo V Source and IonDrive™ High Energy Detector was used. Electrospray ionization was performed in the positive mode (ESI+) with a source temperature of 400°C. Full scan Q1(+), product ion mode MS2(+) and multiple reaction monitoring MRM scan types were used. Typically, a scanning range of m/z ratio between 100-2000 was recorded. Further parameters used for ionization were 4500 V ion spray voltage, an entrance potential (EP) of 10 V, a curtain gas (CUR) with 35 psi, a nebulizer gas (GS1) with 62 psi, a turbo gas (GS2) with 62 psi and a collision gas (CAD) with 10 psi. The instrument was operated in high mass (HM) mode. Peptide solutions of 50 mg/L were analyzed in Q1+ and MS2+ mode. The mass calibration was performed regularly using polypropylene glycol polymer standards (PPGs) (Part no 4406127, AB Sciex, Darmstadt, Germany). For quantification of hepcidin-25 using LC-MS/MS analysis, the triple quadrupole coupled to Agilent 1260 Infinity HPLC system was used, employing chromatographic set 3 and 4 respectively (see *section 3.4.2.2.*). Isotope-labeled hepcidin-25 ("heavy" hepcidin-25) was used as internal standard (IS). Further parameters used to produce fragment ions in multiple reaction monitoring mode (MRM), namely the declustering potential (DP), the collision energy (CE) and the collision cell exit potential (CXP), are presented in Table 2.8. The dwell time was 80 msec and the pause time was 5 msec. One MRM transition was used as quantifier and two transitions as qualifiers respectively, applied to both "light" and "heavy" hepcidin-25 (Table 2.8). The ratio of

the quantifier MRM peak area of "light" hepcidin-25 and "heavy" hepcidin-25 respectively was used for quantification purposes (see *section 2.5.4.*).

Table 2.8: MS/MS transitions with fragmentation parameters for hepcidin-25 and isotope-labeled hepcidin-25 (IS).

Species	Transition (MRM)	DP (V)	EP (V)	CE (V)	CXP (V)	
Hepcidin-25	930.3 → 1144.7 $(y_{21})^{2+}$	40	10	45	6	Quantifier
	930.3 → 1218.2 $(y_{22})^{2+}$	40	10	45	6	Qualifier 1
	930.3 → 354.0 (b_3)	40	10	48	6	Qualifier 2
[$^{13}C_{18}$, $^{15}N_3$] Hepcidin-25	937.3 → 1150.2 $(y_{21})^{2+}$	40	10	45	6	Quantifier
	937.3 → 1228.7 $(y_{22})^{2+}$	40	10	45	6	Qualifier 1
	937.3 → 354.0 (b_3)	40	10	48	6	Qualifier 2

2.4.3.2. Fourier-transform ion cyclotron resonance (FTICR)-MS

The ESI-FTICR Ultra MS (Thermo Scientific, Bremen, Germany) was operated in positive ionization mode (ESI+). A transfer capillary temperature of 200°C was applied. The spectral resolution of the FTICR was set to 1,000,000. Mass calibration was achieved by using a Pierce LTQ ESI Positive Ion Calibration solution. The system was tuned using hepcidin-25 solution 5 mg/L dissolved in water/ACN/TFA 60/38/2 (v/v/v). Typically, a scanning range of m/z ratio between 400-2000 was applied. Direct infusion was employed using the integrated syringe pump set at a flow rate of 30 µL/min.

Hepcidin-25-copper(II) complex solutions (50 mg/L) at pH 11 and 7.4 respectively were analyzed. Volatile solvents/buffers were used as following: The chromatographic starting condition mixture of mobile phase A (pH 11)/mobile phase B 95/5 v/v (chromatographic set 4, see *section 2.4.2.2.*) and 10 mM ammonium acetate (pH 7.4) (see *section 2.2.1.*).

2.4.4. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra were acquired on a Bruker Autoflex III MALDI mass spectrometer (Bruker-Daltonik, Bremen, Germany) operated with a nitrogen laser (355nm, 200 Hz) at 24 kV acceleration voltage in reflectron positive mode (RP+). Mass calibration was performed using Peptide Calibration Standard II (Part no 8222570, Bruker-Daltonik, Bremen, Germany).

A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA), which was used as a matrix, was freshly prepared before the MS analysis. CHCA powder was dissolved in water/ACN/TFA 33/66/0.1 (v/v/v) at a concentration of 10 g/L. The sample preparation was performed using the dried droplet technique (see *section 1.3.2.2.*) on a MALDI target

Steel™ 600/384 (Bruker Corporation, Billerica, USA). One droplet of 0.75 µL of sample (peptide) at a concentration of 10-50 µM was placed on the MALDI target. 0.75 µL of matrix was added to the matrix droplet. The resulting mixture was dried at room temperature. When the liquid has completely evaporated, the sample was loaded into the mass spectrometer. For hepcidin-25 analysis, an m/z ratio between 1000-4000 was applied. The spectra were recorded using 500-1500 laser shots and a laser intensity of 70%.

2.5. Data analysis and treatment

2.5.1. Software

The devices used in the present work were managed by computers running software that allowed for specialized data analysis. As part of this data analysis, the software employs algorithms that convert the detector output signal into qualitative or quantitative data [168]. In this respect, data acquisition and data analysis for HPLC analysis employing Agilent 1260 Infinity Bio-inert was performed using ChemStation® (Agilent Technologies, Waldbronn, Germany). OpenLab® EZChrom (Knauer, Berlin, Germany) was used for chromatographic data treatment using Azura® UHPLC. For experiments using AB Sciex 6500 coupled to Agilent 1260 Infinity, LC-MS data acquisition and analysis were carried out using the software Analyst® 1.6.2 (AB Sciex, Darmstadt, Germany). FTICR-MS spectra have been generated and exported by using Xcalibur® 2.2 (Thermo Fischer Scientific, Dreieich, Germany). MALDI-MS spectra were obtained and analyzed using FlexAnalysis® 1.0 and FlexControl® 1.3 (Bruker-Daltonik, Bremen, Germany). The UV-Vis spectrophotometer was controlled using the software INSIGHT™ 1.4.40 (Thermo Fischer Scientific, Dreieich, Germany).
All calculations of dilution series and concentration determination for sample preparation and LC-MS analysis were conducted by using Excel 2016 and Origin 2017. The graphs and figures were generated and edited by using Origin 2017 and Illustrator CS6.

2.5.2. Exact mass/accurate mass

According to IUPAC definition, **exact mass** refers to the calculated mass of an ion whose elemental formula, isotopic composition and charge state are known using one isotope of each atom involved, usually the lightest isotope. **Nominal mass** refers to the mass of an ion or molecule calculated using the mass of the most abundant isotope of each element rounded to the nearest integer value and equivalent to the sum of the mass numbers of all constituent atoms. **Accurate mass** refers to the experimentally determined mass of an ion measured to an appropriate degree of accuracy and precision [213]. For mass calculations in this work, exact mass will be referred to as theoretical exact mass, and accurate mass

will be referred to as experimental accurate mass or experimentally determined accurate mass. The software Xcalibur® 2.2 was used for theoretical exact mass determinations.

2.5.3. HPLC quantification

The quantification of "in house"-folded hepcidin-25 was performed using HPLC, chromatographic set 3. Seven calibration solutions were measured in the same analytical run in triplicates. The mean HPLC peak area was plotted against the standard concentration. Linear regression was employed for the construction of the calibration curve in the dynamic range 6.25 – 250 mg/L using 7 standards (6.25, 12.5, 25, 50, 100, 150, 250 mg/L). The unknown concentrations of the solutions of hepcidin-25 folded "in house" were calculated from the equation:

$$\text{peak area} = a * \text{concentration} + b,$$

where a and b were determined from the equation of the calibration curve y=ax+b.

2.5.4. ID-LC-MS/MS quantification

2.5.4.1. LC-MS/MS calibration curve

The LC-MS/MS quantification of Hep-25 using TFA-containing mobile phases was performed by employing chromatographic set 3 and was based on a 7-point calibration curve. Similarly, the LC-MS/MS analysis where NH_3-containing solvents were applied used chromatographic set 4 and an 8-point calibration curve. Isotope-labeled hepcidin-25 was used as internal standard (IS). Thus, both external and internal calibrations were applied.
To lower the risk of bias, nine human serum samples and seven sheep calibrators (see *section 2.3.1.*) were measured in the same analytical run three times alternatively as follows:

- calibrators 1-7 1x
- human samples 1-9 1x
- calibrators 1-7 1x
- human samples 1-9 1x
- calibrators 1-7 1x
- human samples 1-9 1x

The mean quantifier MRM peak area of the triplicates was considered further (see *section 2.4.3.1.*). Linear regression was performed on the ratio of the concentrations of "light" and "heavy" hepcidin on the x-axis and the ratio of the corresponding mean MRM peak area of "light" and "heavy" hepcidin on the y-axis. The calibration curve was constructed in the dynamic range 0.5 – 40 µg/L using 7 standards (0.5, 1, 2, 5, 10, 20, 40 µg/L) and 8 standards respectively (0.3, 0.5, 1, 2, 5, 10, 20, 40 µg/L).

2.5.4.2. International Hepcidin Harmonization Study (HHS2017)

The international Hepcidin Harmonization Study (HHS2017) for the harmonization of hepcidin-25 measurement results was organized by Radboud University Nijmegen, the Netherlands, and hepcidinanalysis.com as a follow-up to previous steps taken towards harmonization (see *section 1.3.3.*). The aim was to perform a proof of principle study on hepcidin harmonization among various worldwide leading hepcidin assays by using a secondary commutable lyophilized reference material. A calibration set and three human serum samples were delivered from Radboud University Nijmegen. The organizers requested the quantification of the unknown human samples with the assay calibrated with and without the calibration set. The quantification was performed using LC-MS/MS (chromatographic set 3, TFA-based mobile phases) by employing two calibration methods in separate analytical runs:

a. 7-point calibration (without HHS2017 calibration set)

For method calibration, seven sheep calibrators were prepared and linear regression was performed for a 7-point calibration curve as presented in *section 2.5.4.1.* Isotope-labeled hepcidin-25 was used as internal standard.
Three human serum samples and seven calibrators were measured in the same analytical run three times alternatively as presented in *section 2.5.4.1.*

b. 2-point calibration (with HHS2017 calibration set)

For method calibration, a secondary reference material developed by hepcidinanalysis.com (Nijmegen, the Netherlands) was used and linear regression was performed for a 2-point calibration curve. Isotope-labeled hepcidin-25 was used as internal standard. The calibrator set contained pooled lyophilized native human serum with the addition of stabilizer CLP (Chymotrypsin Like Proteinase) and consisted of one “HHS2016 calibrator low level” and one “HHS2016 calibrator middle level”, with values assigned from the calculated mean value of 9 validated methods worldwide [214] (Table 2.9).
Three human serum samples and two calibrators were measured in the same analytical run in triplicates, according to the received instructions. The calibrators were measured at the start, middle and end of the sample run as follows:

- calibrators 1-2 3x
- human samples 1 3x
- calibrators 1-2 3x
- human samples 2 3x
- calibrators 1-2 3x
- human samples 3 3x

Similarly as in the 7-point calibration, linear regression was performed on the ratio of the concentrations of "light" and "heavy" hepcidin on the x-axis and the ratio of the corresponding mean MRM peak area of "light" and "heavy" hepcidin on the y-axis. A 2-point calibration curve was constructed for each human sample based on the calibrator set analyzed beforehand. A total of three calibration curves were obtained and the unknown concentrations of the human samples were calculated from their correspondent regression line.

Table 2.9: Assigned values for calibrator set (hepcidinanalysis.com).

Level	Mean value (SD) nmol/L
Low	2.38 (1.22)
Middle	7.03 (3.15)

2.5.4.3. Concentration calculations

The isotope-labeled hepcidin-25 is used for internal calibration and the sheep serum calibrators/calibrators set as external calibration. The quantification is based on using the ratio of the internal calibration to extrapolate the unknown concentrations from the calibration curve (external calibration). In this sense, the calibration curve (7-point or 2-point) resulted in the formula y=ax+b. The parameters a and b were calculated. The unknown concentration of the human serum samples (concentration lH) was calculated from the formula:

$$\frac{\text{peak area MRM lH}}{\text{peak area MRM hH}} = \text{a} * \frac{\text{concentration lH}}{\text{concentration hH}} + b$$

where lH = "light" hepcidin-2 and hH = "heavy" hepcidin-25.

2.6. LC-MS/MS validation

The validation of the LC-MS/MS methods was carried out in accordance to the LC-MS guidelines of the Clinical and Laboratory Standards Institute (CLSI) [168] regarding specificity, linearity, precision, accuracy, recovery and matrix effects, and the European directives 2002/657/EC concerning the performance of analytical methods and the interpretation of results [83] in terms of relative ion intensities in MRM mode. The acceptance criteria were defined accordingly based on the mentioned guidelines.

Specificity

Specificity was defined as the ability of a method to measure solely the analyte of interest [168]. This was assessed by verifying the absence of interference peaks in the surrogate-matrix at the same retention times as light and heavy hepcidin-25. For this purpose, bovine and sheep serum were tested as a surrogate matrix (free of human hepcidin-25). Two MRM transitions were used as qualifiers for both "light" and "heavy" hepcidin-25 (see *section*

3.4.3.1.). The maximum permitted tolerance for relative ion intensities (% MRM qualifier/MRM quantifier) of >20% to 50%, as in the case of both "light" and "heavy" hepcidin-25, was ± 25% [83].

Linearity

Linearity was defined as the ability of the method to provide measured quantity values that are directly proportional to the value of the analyte in the sample within a given measuring interval [168]. In this regard, the assessment of the calibration curve was performed on seven sheep serum standards (0.5, 1, 2, 5, 10, 20, 40 µg/L) for the LC acidic conditions with an additional standard at 0.3 µg/L for the LC basic conditions. A linear regression analysis was performed to set up the calibration line within the dynamic range of 0.5-40 µg/L and 0.3-40 µg/L respectively. Serial dilutions to create a linearity set was avoided, due to additive pipetting errors that can introduce systematic errors [168].

Precision and accuracy

Precision was defined as closeness of agreement between measurement results obtained under stipulated conditions [168]. Intra- and inter-assay precision were determined. Intra-assay precision or repeatability expressed the precision under the same operating conditions over a short interval of time (same analytical run). Inter-assay precision or intermediate precision expressed within-laboratory variations in consecutive days (different analytical runs). Precision was expressed as coefficient of variation (CV) and calculated as percentage relative standard deviation (% RSD = standard deviation/ mean · 100).

Accuracy was defined as closeness of agreement between a measured quantity value and a true quantity value of the analyte [168]. In the absence of reference materials, the conventional true value was considered the nominal value of analyte reported by the supplier for "light" and "heavy" hepcidin-25 (see *section 2.3.1.1.*). Accuracy was calculated as percentage relative error (% RE = [(value found/ conventional value) – 1] · 100).

[[$^{13}C_9$,^{15}N]Phe^{4,9},[^{15}N]Gly^{12}]-Hepcidin-25 was used as IS to compensate for instrumentation variables.

Five sheep serum standards were considered over the quantification range (0.5-40 µg/L) for the assessment of intra-assay precision and accuracy. The samples were measured in triplicates within the same day. Three quality control (QC) samples at high (25 µg/L), medium (10 µg/L) and low (2 µg/L) level were chosen for the determination of inter-assay precision and accuracy. The samples were measured in five replicates in two consecutive days. According to CLSI guidelines [168], the imprecision of the concentration should not exceed 15% CV except for the LOQ, where ≤ 20% CV is acceptable. Also, an accuracy <±15% is considered satisfactory.

LOD, LOQ

The limit of detection (LOD) was defined as the lowest concentration at which the signal-to-noise ratio S/N was higher than 3:1. The limit of quantification (LOQ) was determined in an analogous way for S/N greater than 10:1 [168].

Matrix effect and recovery

The matrix effect was defined as the MRM signal suppression caused by the matrix (sheep serum). The occurrence of matrix effects was tested by comparing the peak areas of two QC samples (5, 10 µg/L) in neat solution and sheep serum respectively [168]. The samples in neat solutions were prepared by diluting hepcidin-25 standards to the desired concentration using a mixture of water/ACN/TFA 60/38/2 v/v/v (see *section 2.3.1.1.*). The samples in sheep serum were prepared as described in Protocol I and Protocol II respectively (see *section 2.3.1.2.* and *2.3.1.3.* respectively). The recovery was defined as the yield of the extraction and/or pre-concentration step for the analyte divided by the amount of analyte in the original sample. Two QC samples (5, 10 µg/L) were used for the assessment of recovery by comparing peak areas of the quantifier MRM for measuring sheep serum samples spiked before and after sample preparation respectively [215, 168]. The sheep serum samples spiked before sample preparation were handled as described in Protocol I and Protocol II respectively (see *section 2.3.1.2.* and *2.3.1.3.* respectively). In the case of the samples spiked after sample preparation, "light" hepcidin-25 (50 µg/L) was added to the supernatant obtained after sample preparation to achieve the desired concentration. Generally, the IS ([[$^{13}C_9$,^{15}N]Phe^{4,9},[^{15}N]Gly^{12}]-Hepcidin-25) was spiked in the early stage of the sample preparation, to compensate for losses during sample handling and for matrix effects [168]. For a good sensitivity of the method, the allowed magnitude of matrix effects was defined as < 35% signal suppression and a recovery > 95% was considered acceptable.

3. Results and Discussions

3.1. Characterization of the physico-chemical properties of hepcidin-25

Hepcidin-25 is a 25-residue peptide with the following amino acid sequence DTH-FPICIFCCGCCHRSKCGMCCKT (Figure 3.1), which forms a distorted β-sheet with a flexible N-terminus. The eight cysteine residues are involved in four disulfide bridges, namely between Cys7-Cys23 (1-8), Cys10-Cys13 (2-4), Cys11-Cys19 (3-6) and Cys14-Cys22 (5-7) [85] (see *section 1.2.2.*), with the resulting chemical formula $C_{113}H_{170}N_{34}O_{31}S_9$.

Figure 3.1: Amino acid sequence of hepcidin-25 containing acidic amino acids (red) and basic amino acids (blue).

Hep-25 contains aspartic acid D as acidic residue and histidine H, arginine R and lysine K as basic residues (Figure 3.1), with an additional acidic -COOH moiety at the C-terminus and a basic N-terminal amino group. Having an approximate net charge of +4 at neutral pH, hepcidin-25 is a basic peptide, with an isoelectric point of 8.2 [146]. Also, Hep-25 shows an amphipathic character due to the presence of hydrophilic residues (e.g. lysine K, arginine R, histidine H, aspartic acid D) and hydrophobic uncharged residues (e.g. leucine L, isoleucine I, methionine M, proline P, phenylalanine P).

The amino acid content of Hep-25 together with the complexity of the disulfide network influences the physico-chemical behavior of the peptide and will be discussed further. In this regard, the folding process of hepcidin-25 will be presented, followed by solubility tests and conjugation of the peptide. Furthermore, the copper binding capacity of hepcidin-25 due to the presence of the ATCUN motif will be addressed.

3.1.1. Production of folded hepcidin-25

The folding of hepcidin-25 can be challenging and miss-folding artifacts can occur because of the complex network of disulfide bonds [85]. Two different assignments of the intramolecular disulfide bridges by Hunter *et al.* in 2002 and by Jordan *et al.* in 2009 (see *section 1.2.2.*) indicate that the folding of linear hepcidin-25 is a critical issue in the analysis of the peptide. The unanimously accepted correct disulfide connectivity of hepcidin-25 is nowadays considered the version published in 2009 between Cys7 and Cys23 (1-8), Cys10 and Cys13 (2-4), Cys11 and Cys19 (3-6), and Cys14 and Cys22 (5-7) [85]. Although the vendors who commercialized synthetic human hepcidin-25 before 2009 reported the "wrong" connectivity (2002), it is unclear if the peptide ever presented two different folding patterns. Recent ion mobility spectrometry

(IMS) experiments performed by Bros *et al.* on two hepcidin-25 standards obtained from different synthesis pathways determined several compounds with different mobility profiles (size-to-charge ratios) in the composition of the standards. The authors suggested that these compounds could represent different folding products resulted from different synthesis protocols, which could indicate that the folding protocol can lead to conformational variety [87]. However, the products obtained in the IMS experiments could not be further identified and no disulfide connectivity was attributed. Also, no details about the synthesis protocols were provided. The need remains for effective folding methods to be developed that can be correlated to the currently accepted disulfide connectivity. Thus, the present work investigated the folding possibilities of the linear hepcidin-25. Furthermore, a reliable route for the "in house" preparation of folded hepcidin from the linear peptide was developed. In order to characterize the different possible folding products obtained, HPLC was chosen as an analytical technique able to differentiate between different conformers.

Three different folding conditions available in the literature were compared. By such, linear hepcidin-25 was incubated (once at a time) in reduced-oxidized glutathione solution at pH 7.4 (Jordan *et al*. [85]), cysteine-cystine-guanidine-EDTA-PBS buffer at pH 7.8 (Clark *et al*. [92]) and HEPES/urea mixture at pH 7 (Tselepis *et al.* [107]) respectively. The complete formation of the intramolecular disulfide bonds was carried out overnight (approximately 20 hours). HPLC analysis of the resulting solution showed one sole folding product in all three cases, characterized by a retention time corresponding to the commercial synthetic human hepcidin-25 (data not shown). It could be concluded that human hepcidin-25 presents one disulfide connectivity that is thermodynamically stable and can result from the oxidation of the linear 25-residue peptide. This came in agreement with previously reported findings where different folding protocols applied led to a single folded product [211]. The reduced-oxidized glutathione-based folding buffer was chosen out of the three folding conditions to further investigate and characterize the folding process.

3.1.1.1. HPLC characterization

The commercial linear (reduced) human hepcidin-25 was folded applying an adapted protocol from Jordan *et al.* as presented in *section 2.2.3*. The authors indicated an oxidized-reduced glutathione mixture at a pH of 7.4. However, lowering the pH value proved to increase the amount of folded peptide obtained (data not shown), probably due to the increase in the solubility caused by moving the pH value away from the isoelectric point of 8.2, and thus increasing the charge of the peptide. Therefore, the folding of linear hepcidin-25 was performed at a pH of 7. The use of organic solvent (ACN) and acid (TFA) was also found necessary to enhance the solubility of the peptide (see *section 3.1.2.*).

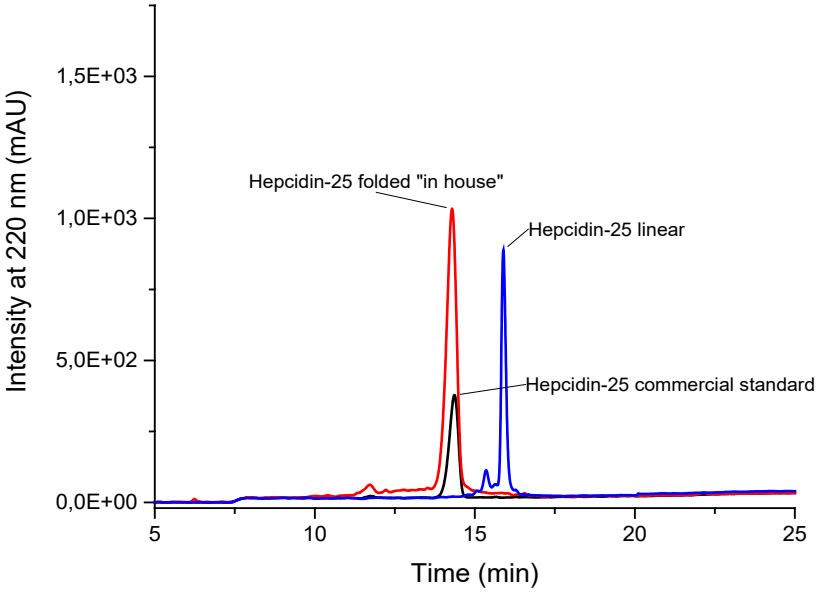

Figure 3.2: Hepcidin-25 species (HPLC) at 220 nm: 25 µM hepcidin-25 commercial standard (black trace), 50 µM linear hepcidin-25 (blue trace), 50 µM hepcidin-25 folded "in house" (red trace).

The commercial hepcidin-25 standard (folded), the commercial linear hepcidin-25 and the folding product underwent HPLC analysis (Figure 3.2). The commercial peptides were analyzed in an aqueous solution of 50 µM (linear hepcidin-25) and 25 µM (folded hepcidin-25). The "in house" folded species was injected at a concentration of approximately 50 µM (140 mg/L) in the folding buffer. The hepcidin-25 standard (oxidized) showed an eluting peak characterized by a retention time of 14.3 minutes, while the reduced species is more hydrophobic due to presence of the free -SH groups and eluted later at a retention time of 15.9 minutes. It could be observed that the "in house" folded hepcidin-25 co-eluted with the commercial standard, displaying an identical retention time of 14.3 minutes. At this point it could be concluded that a folding procedure of hepcidin-25 was developed which yielded a sole product. This presented identical chromatographic behavior as the commercial hepcidin-25 standard, which reports the currently accepted disulfide connectivity between Cys7 and Cys23 (1-8), Cys10 and Cys13 (2-4), Cys11 and Cys19 (3-6), and Cys14 and Cys22 (5-7) [85]. MS characterization by MALDI-TOF confirmed the mass and isotope pattern of the folded product to be identical to the commercially available standard (see *section 3.1.1.2.*).

Further, with the aim to obtain multi-milligram quantities of oxidized hepcidin-25, the folding protocol was examined using various amounts of linear hepcidin, by scaling up the process from low (33 mg/L) to high (2 g/L) peptide concentration. Unfortunately, highly concentrated solutions of linear hepcidin showed very poor folding yields. This could be explained by the aggregation tendency of the peptide [84] that might form three-dimensionally cross-linked pol-

ymers at high concentrations. The use of reducing agents such as Tris-(2-carboxyethyl)-phosphine (TCEP) and of folding enzymes such as protein disulfide isomerase (PDI) did not have any effect on the polymers formed (data not shown).
The concentration of 33 mg/L was selected to achieve good yields of the folding process. For practical reasons, not more than 1 mg linear hepcidin was used, for which 30 mL of folding solution were required. Semi-preparative HPLC was employed for the purification of the folding product (chromatographic set 1, see *section 2.4.2.2.*). The peptide solutions were stored in a mixture of water/ACN/TFA 60/40/0.1 (v/v/v) at 4°C and used within a month from preparation. The quantification of the obtained oxidized peptide and the yield of the folding process will be discussed in *sections 3.1.1.3.* and *3.1.1.4.,* respectively.

3.1.1.2. MS characterization

The three hepcidin-25 species (commercial standard, commercial linear peptide and "in house" folded peptide) were analyzed using MALDI-TOF-MS for mass determinations. The sample preparation was described in detail in *section 2.4.4.*

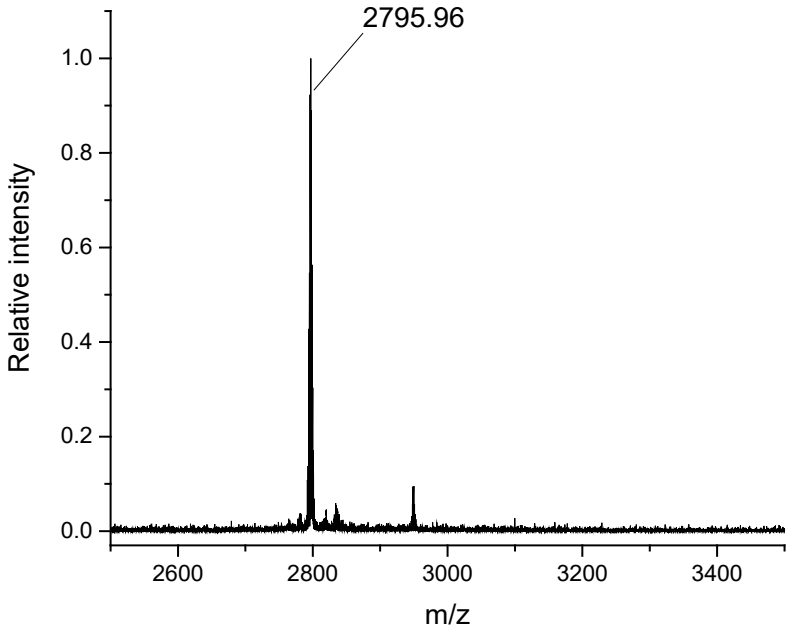

Figure 3.3: MALDI-TOF-MS spectra of commercial linear hepcidin-25.

The theoretical exact mass of hepcidin-25 was 2787.02576 Da for the folded species and 2795.0884 Da for the linear species respectively (see *section 2.5.2.*). The difference of 8 Da is represented by the loss of 8 hydrogen atoms due to the formation of the four disulfide bridges. In case of linear hepcidin-25, the manufacturer stated a purity of >95% assessed by amino acid analysis. MALDI-TOF characterization of a solution of 50 µM linear hepcidin-25 revealed a $[M+H]^+$ of 2795.96 (Figure 3.3), which is in agreement with the theoretical exact mass of 2795.0867150, considering a resolution of the device of approximately 0.1 Da. It was concluded

that the peptide is suitable to be used further in the production of folded hepcidin-25. Further, the commercial standard (purity >95% assessed by HPLC analysis) was compared with the "in house" folded peptide. Similarly, as in the HPLC analysis, an aqueous solution (1% acetic acid) of 25 µM commercial folded hepcidin-25 and a 50 µM solution of "in house" folded species in folding buffer were analyzed.

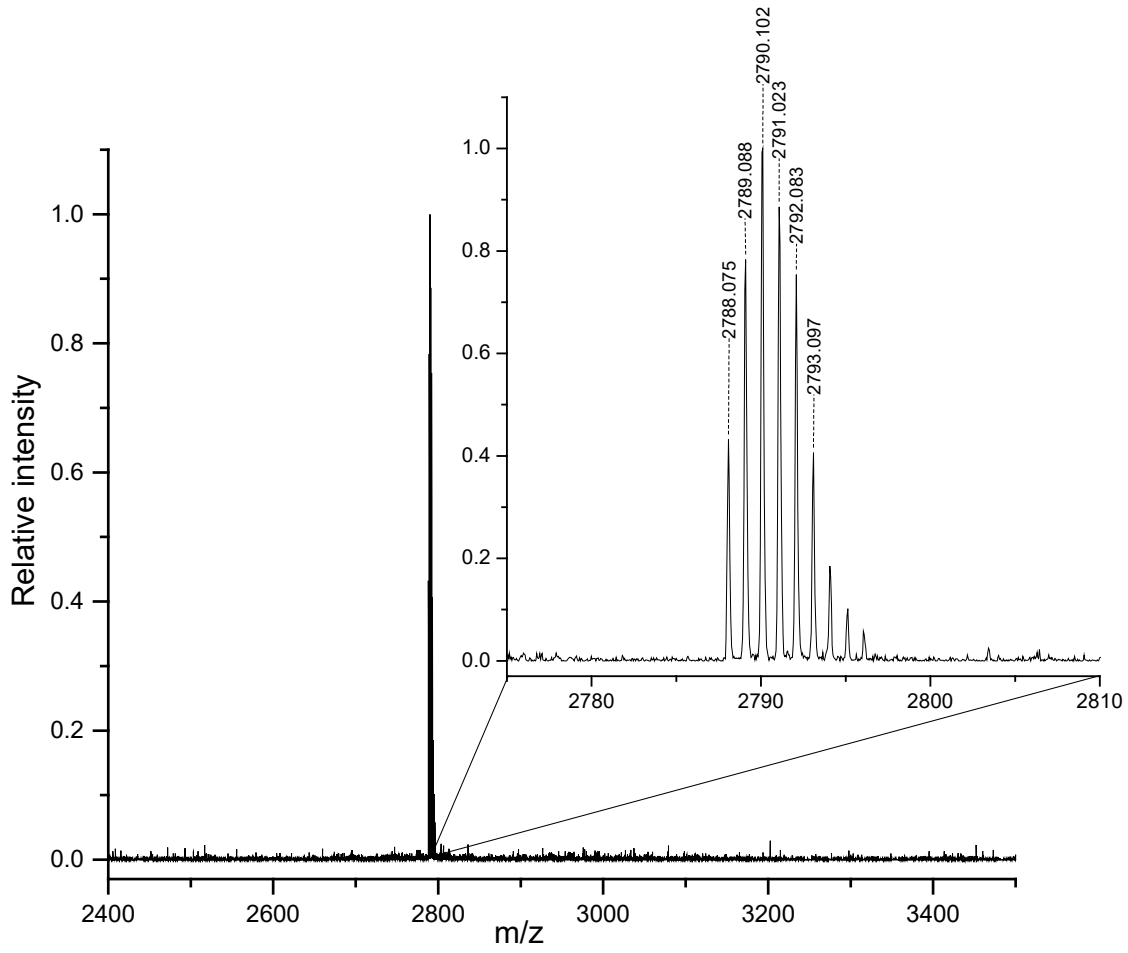

Figure 3.4: MALDI-TOF-MS spectra of commercial hepcidin-25 (folded).

The MS spectra of the commercially purchased standard and the "in house" folded hepcidin-25 showed a very similar isotope pattern, with a $[M+H]^+$ of 2788.08 and 2788.10 respectively (Figures 3.4 and 3.5 respectively). This came in agreement with the theoretical exact mass of 2787.0252 Da of human hepcidin-25.

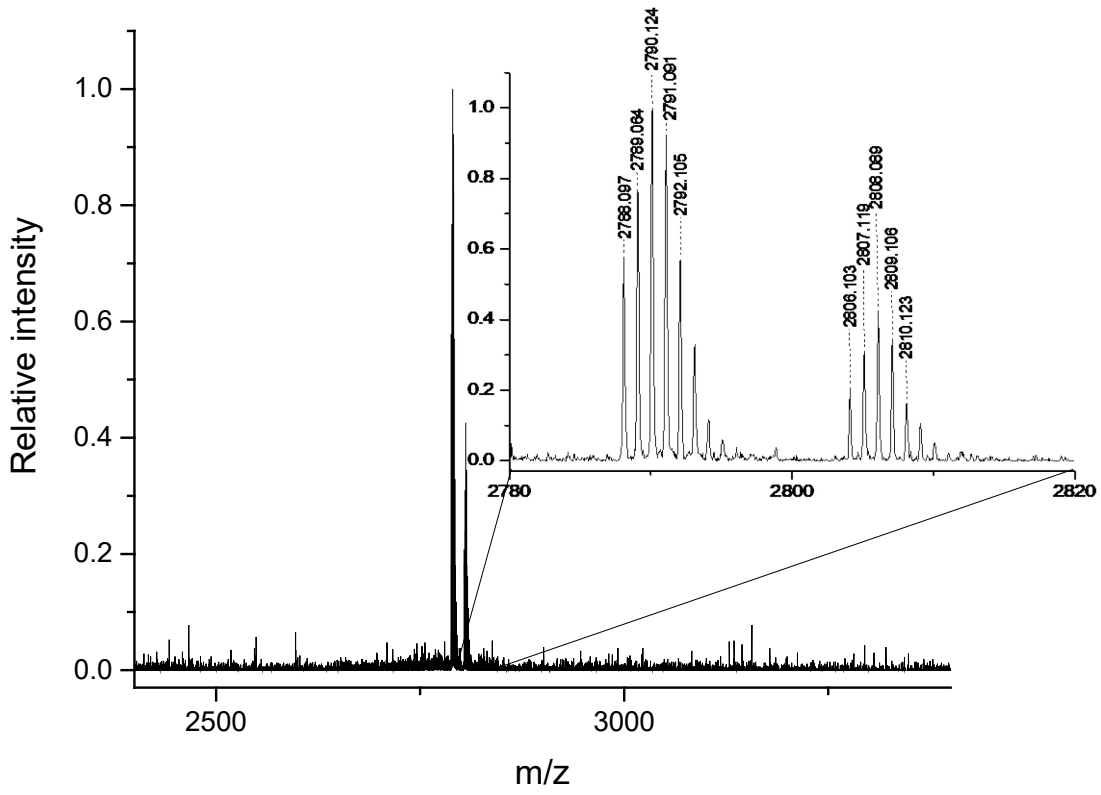

Figure 3.5: MALDI-TOF-MS spectra of hepcidin-25 folded "in house".

Figure 3.5 shows the MS spectra of the "in house" folded peptide, analyzed before purification. One single folding product could be identified as corresponding to the mass of hepcidin-25 ($[M+H]^+$ 2788.10). The spectra also indicated a MS peak corresponding to a mass of 2806.10 Da. The mass difference of 18 Da indicated that this peak correlated to the ammonium adduct of hepcidin-25 (folded) $[M+NH_4]^+$. This could be explained by the addition of ammonium hydroxide to the folding buffer for pH adjustment (see *section 2.2.1.*).

3.1.1.3. Quantification of folded hepcidin-25

The high amounts of purified "in house" folded hepcidin-25, obtained in the concentration range of 20-250 mg/L, permitted a simple and quick quantification of the peptide by reversed-phase (RP)-HPLC, chromatographic set 3 (see *section 2.4.2.2.*). A calibration curve was constructed using 7 standards in the dynamic range of 6.25 - 250 mg/L, by plotting the HPLC peak area of the standards versus standard concentration (Figure 3.6). Applying a quantification approach including external calibration and without the use of an internal standard was considered suitable for the assessment of the folding yield in the selected dynamic range.

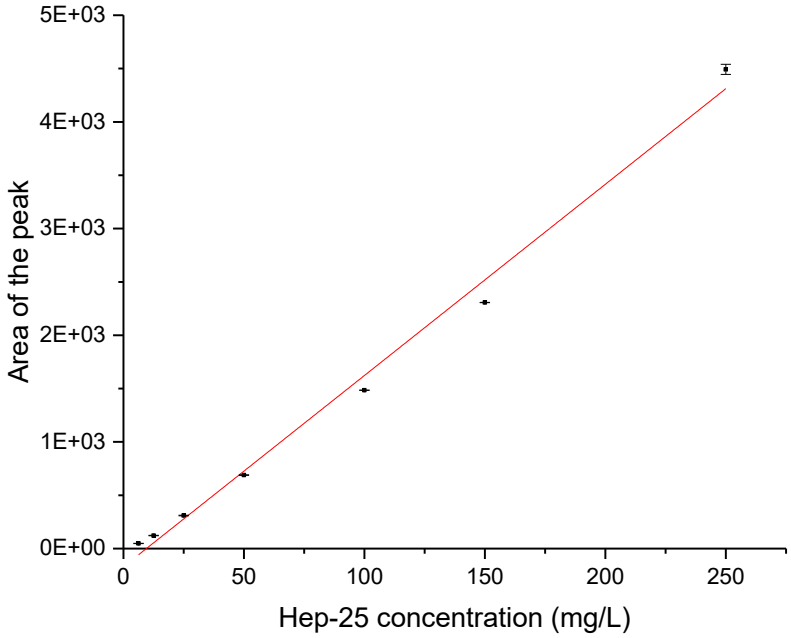

Figure 3.6: 7-point calibration curve (y = 17.928x - 170.170, r^2 = 0.999) by HPLC.

The average correlation coefficient (r^2) was 0.999. A very good intra-assay precision (<3%) was achieved (Table 3.1). A LOQ of 6.25 mg/L was obtained for the quantification of hepcidin-25 using HPLC.

Table 3.1: Intra-assay precision (n=3) defined as coefficient of variation (CV).

Hep-25 concentration (mg/L)	CV (%)
250	1.1
100	0.1
25	1.0
6.25	2.9

3.1.1.4. Yield of the folding process

The yield of the folding process was determined by considering the amount of linear hepcidin-25 subjected to folding and the amount of oxidized peptide obtained. In this sense, the formula below was applied:

$$\text{Yield (\%)} = \frac{amount\ of\ folded\ hepcidin{-}25\ (mg)}{amount\ of\ linear\ hepcidin{-}25\ (mg)} * 100$$

The amount of linear hepcidin-25 used was 1 mg each time. The amount of folded Hep-25 was determined after purification using RP-HPLC. The total yield characterized the folding and the purification processes. A peptide purity of >95% was achieved.

Good yields were achieved using a concentration of linear hepcidin of 33 mg/L (10-50%) (Table 3.2). The values were similar or even higher as compared to previously reported yields (12%) [211]. A mean yield of approximately 25% was obtained. This indicated an amount of 2.5 mg “in house” folded peptide obtained from 10 mg linear hepcidin-25. No correlation could

be assigned between the folding time and the yield of the process. Any folding duration between 16 and 24 hours seems to be suitable for the complete folding. In this context, it needs to be mentioned that an investigation of partial folding was not the aim of the study. The standard deviation of approximately 13% for the calculated yields indicated an average reproducibility of the folding process. The main source of variability was probably the purification process when fluctuating losses of folded hepcidin-25 could have arisen while loading the folding solution onto the semi-preparative C18 column (see *section 2.2.3.*).

Table 3.2: Calculated total yields for the folding process (concentration of linear hepcidin-25 of 33 mg/L).

Folding time (h)	Total yield* (%)
22	19
19	32
19	22
17	9
17	48
17	44
17	11
16	20
16	39
16	20

*hepcidin-25 purity >95% (HPLC analysis)

The purchase of linear hepcidin-25 was found to be 20-fold more cost effective compared to that of the hepcidin-25 standard (folded). The development of an "in house" folding method offered not only insights into the folding process, but also delivered a considerably higher amount of peptide that could be used for various experiments within the context of this work.

3.1.2. Solubility of hepcidin-25

Generally, the amino acid content determines the solubility of the peptides in water, solvents and buffers at different pH values. Hepcidin-25 comprises of a high number of basic residues and shows an amphipathic character, with both hydrophilic and hydrophobic amino acids contained in its structure. To test the solubility of hepcidin-25, initially, the peptide was dissolved in water. Further, acidic solutions and polar solvents were used to solubilize hepcidin-25. Usually, acetic acid or stronger acids such as TFA are used for basic peptide solubilization. Then, various polar solvents were applied. Methanol and acetonitrile were selected because they represent solvents compatible with LC-MS analysis. Ultimately, various buffers were tested to assess the solubility of the peptide close to the physiological pH (pH range of 7.4 to 8.3). For this purpose, 50 µL of 130 mg/L hepcidin-25 folded "in house" (65 µg) were dried in the vacuum oven (see *section 3.2.5.*). Then, various reconstitution solvents were tested as described above. HPLC analysis was applied to determine the peptide solubility by comparison of the

HPLC peak areas. As expected, the solubilization of the basic peptide was facilitated by acidic solvents such as acetic acid or TFA. The solubility in water was lower in comparison to other polar solvents or acidic solutions. ACN, methanol, acetic acid and TFA seemed to be good solvents for the peptide. The increase in concentration of the polar solvent (methanol, acetic acid) led to an increase in the solubility of the peptide. However, a mixture containing ACN and TFA seemed to be the best solvent for hepcidin-25. Water/ACN/TFA 60/40/0.1 (v/v/v) showed a higher solubilization capacity compared to 100% methanol or 100% acetic acid (Figure 3.7). The choice for the optimum ratio of water/ACN/TFA will be considered in more detail in *section 3.3.3.1.*

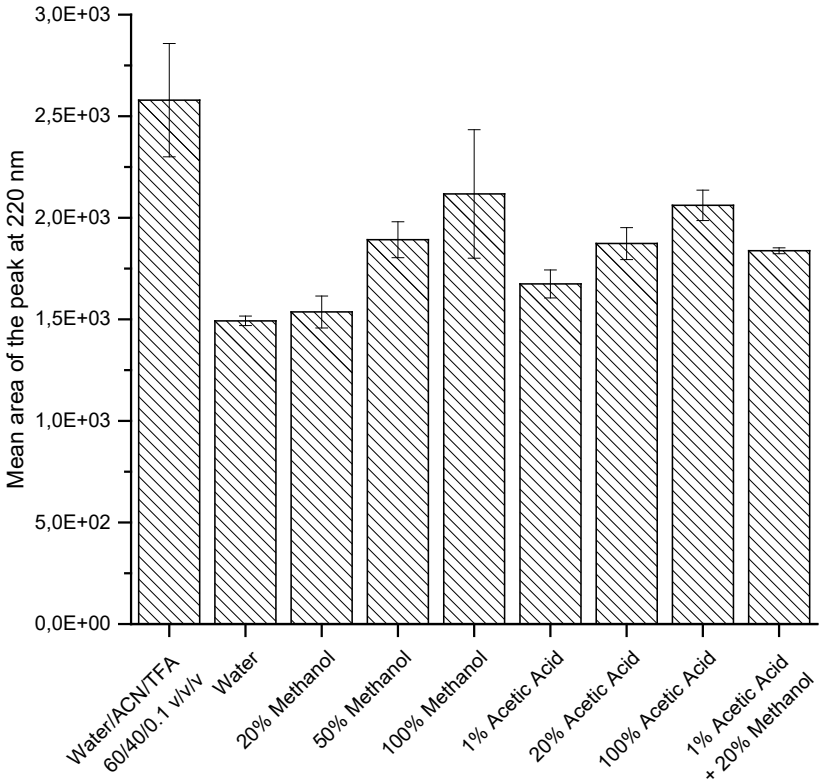

Figure 3.7: Area of the peak (HPLC analysis) for hepcidin-25 solutions in different solvents.

Further, the peptide showed poorer solubility in all the aqueous buffers in the pH range of 7.4 to 8.3 compared to water (and other organic solvents). As expected, the solubilization capacities of the buffers decreased in the proximity of the isoelectric point (8.2). Ammonium acetate buffer at a pH of 8.3 or borate buffer at a pH of 8 were the least effective solvents compared to sodium bicarbonate buffer at a pH of 7.8 or PBS buffer at a pH of 7.4. However, an addition of 50% of methanol visibly increased the solubility of hepcidin-25 (Figure 3.8).

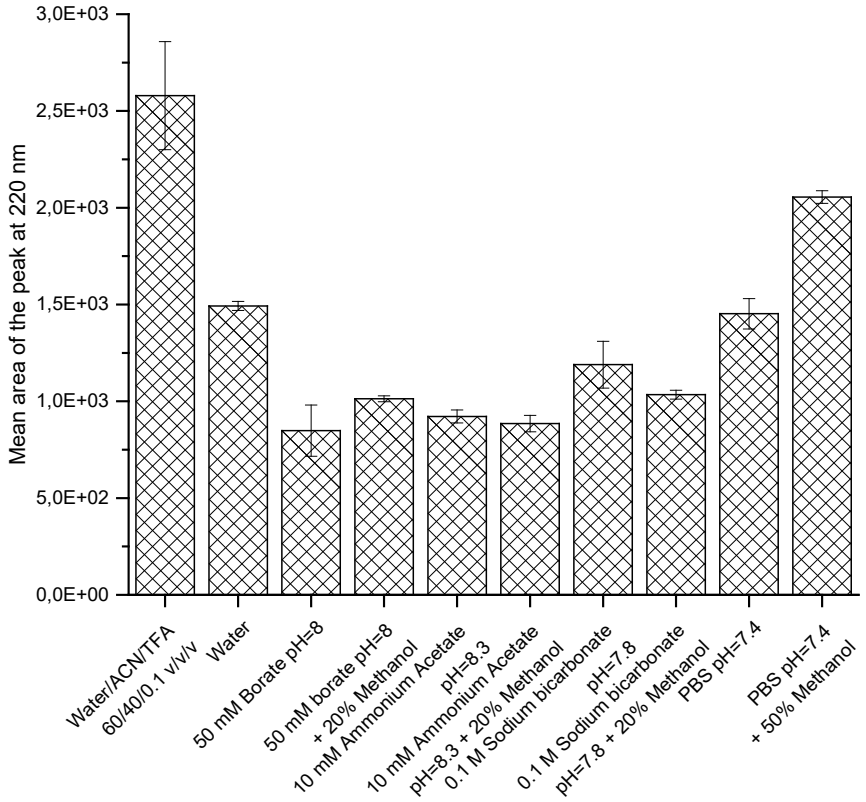

Figure 3.8: Area of the peak (HPLC analysis) for hepcidin-25 solutions in different buffers and solvent-buffer mixtures.

As a conclusion, a guideline in preparing hepcidin-25 solutions was established. The peptide was most soluble in acidic solutions (1% acetic acid or 0.1% TFA) using polar solvents (40% ACN). Various buffers (in the pH range of 7.4 to 8.3) did not show very good solubilization capacity, however their effectiveness was greatly enhanced by the addition of higher concentrations of polar solvents (e.g. 50% methanol).

3.1.3. Synthesis of labeled hepcidin-25 with a fluorescent dye

Labeled hepcidin-25 species could be extremely valuable for different quantification purposes or in bioactivity assays. The lack of suitable immunoassays for hepcidin-25 quantification (see *section 1.3.1.* and *1.3.3.*) can partly be because of challenging production of hepcidin-25 conjugates, particularly of the mono-labeled peptide. Due to the compact structure of hepcidin-25 and its tendency to aggregate, especially around the isoelectric point of 8.2 which is usually the required pH value for bioconjugation, the synthesis of such conjugates can be difficult.

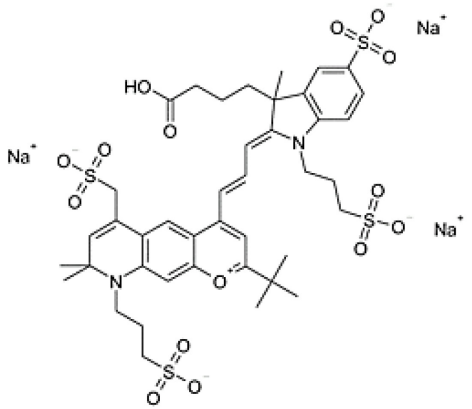

Figure 3.9: Structure of the fluorescent dye DY-654 [216].

Moreover, hepcidin-25 presents three amino groups that could participate in conjugation reactions, namely one -NH_2 group at the N-terminus and two amino groups within the lysine residues. Therefore, the synthesis of a mono-labeled peptide requires careful choice of the pH and possibly a separation step from multi-labeled peptide species.

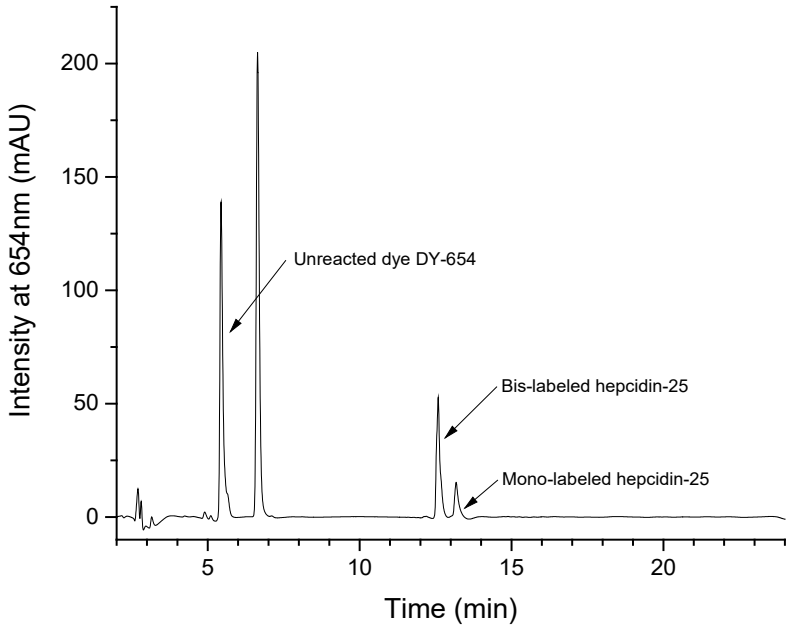

Figure 3.10: HPLC chromatogram of the conjugation products of hepcidin-25 (654nm).

The synthesis of fluorescent mono-labeled hepcidin-25 was performed using the N-hydroxy succinimid (NHS) ester of DY-654 (Figure 3.9), a dye soluble in water and methanol, with a maximum absorption/emission at the wavelengths 654 nm/677 nm respectively. NHS ester-activated labeling compounds react with primary amines in the pH range of 7.2 to 9 to yield stable amide bonds [147, 148].

As a consequence, two types of labeled hepcidin-25 species were obtained. These represent a mono-labeled and a bis-labeled peptide. The purification of the two species was performed by HPLC using chromatographic set 2 (see *section 2.4.2.2.*). Figure 3.10 shows the chromatogram at 654 nm indicating four compounds. The MS analysis of the obtained products identified the hydrolyzed unreacted dye (free acid) at the minute 5.4 with a mass of 942 Da, and the mono- and bis-labeled hepcidin-25 species at minute 12.6 and 13.2 respectively. The compound at minute 6.6 could not be attributed. The MS spectra of the two fluorescent-labeled hepcidin-25 species showed the triply and quadruply charged quasi-molecular ions. A mass of 4635 Da was recorded for the species containing two labels ($[M+4H]^{4+}$ of 928.7 and $[M+3H]^{3+}$ of 1237.6 as shown in Figure 3.11), which correlates to one molecule of Hep-25 and two molecules of fluorescent dye with the loss of two water molecules (2787+2·942-2·18). Moreover, a mass of 3711 Da was obtained for the mono-labeled peptide ($[M+4H]^{4+}$ of 1159.2 and $[M+3H]^{3+}$ of 1545.1 as presented in Figure 3.12) which corresponds to one molecule of hepcidin-25 and one molecule of dye DY-654 with the loss of one molecule of water (2787+942-18).

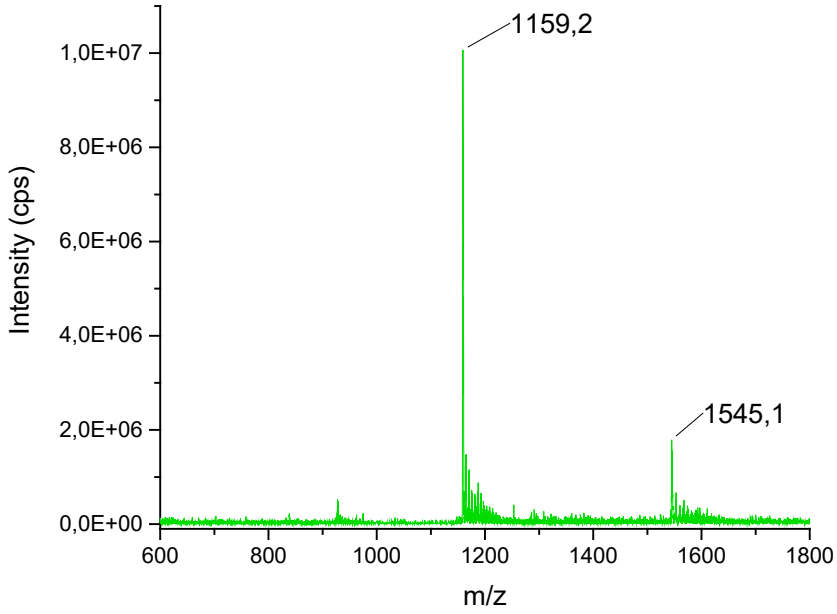

Figure 3.11: ESI-MS spectra of bis-labeled hepcidin-25.

Other fluorescent-labeled hepcidin-25 species have also been reported in literature using Texas Red-X dye, however no structural information such as the product mass or number of labels has been provided [117].The synthetized hepcidin-25 with a fluorescent tag can be applied in anti-hepcidin-25 antibody screening protocols with fluorescence detection.

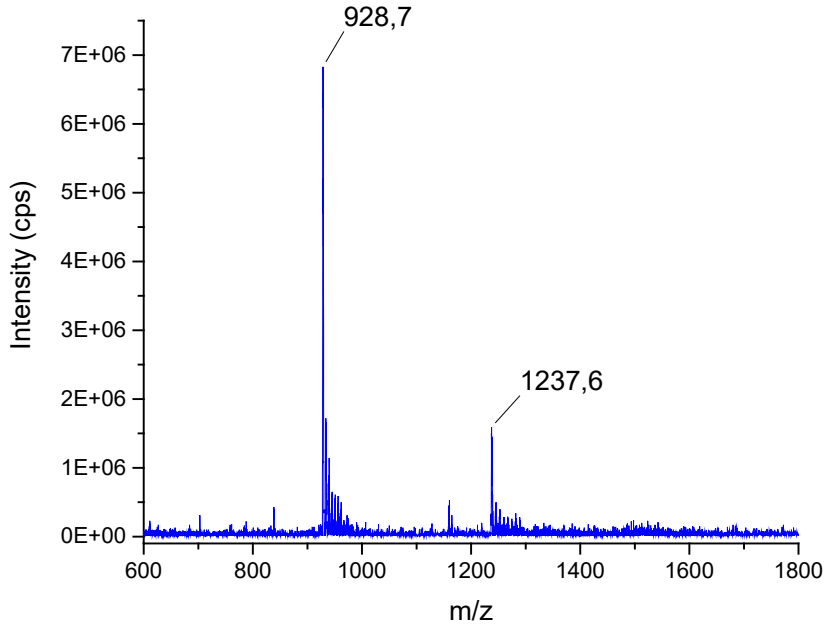

Figure 3.12: ESI-MS spectra of mono-labeled hepcidin-25.

3.2. Structural considerations of hepcidin-25-copper(II) complexes

The presence of the ATCUN motif, a small metal binding site in the structure of hepcidin-25 is responsible for the high affinity of the peptide for Cu(II) ions (see *section 1.2.3.*). Enzymes and proteins that contain copper binding sites include copper both in their structure and biological function. As an example, human serum albumin HSA exhibits its function in copper transport due to the ATCUN motif present at the N-terminus (see *section 1.2.3.1.*). This indicates that the presence of a copper binding site in the structure of a peptide or protein should not be ignored. Generally, the affinity of the ATCUN motif for copper can be explained by the presence of nitrogen donors that attract copper ions with the simultaneous formation of three connected chelate rings. Cu(II) is one of very few metal ions able to deprotonate and coordinate the peptide bond nitrogen. Moreover, binding with the N_{δ} of the histidine side chain creates a stable 6-membered chelate ring with the preceding amide nitrogen. Also, the amino group at the N-terminus anchors the metal in a 5-membered chelate which contributes to the stability of the complex [110]. The 4N metal complexes (see *section 1.2.3.1.*) formed in this way seem to be the most stable ones formed by peptides [217]. Furthermore, reports showed that the presence of aspartic acid (D) as residue 1 in the ATCUN motif increases the basicity of the nitrogen atoms involved in the metal complex and thus enhancing the copper binding [102].
Investigations by Plonka *et al.* of the N-terminus of hepcidin-25 (DTHFPI) resulted in the highest affinity of an ATCUN motif for copper ever reported. Additionally, the 6-residue peptide could exchange the copper ions from HSA [112] (see *section 1.2.3.2.*).

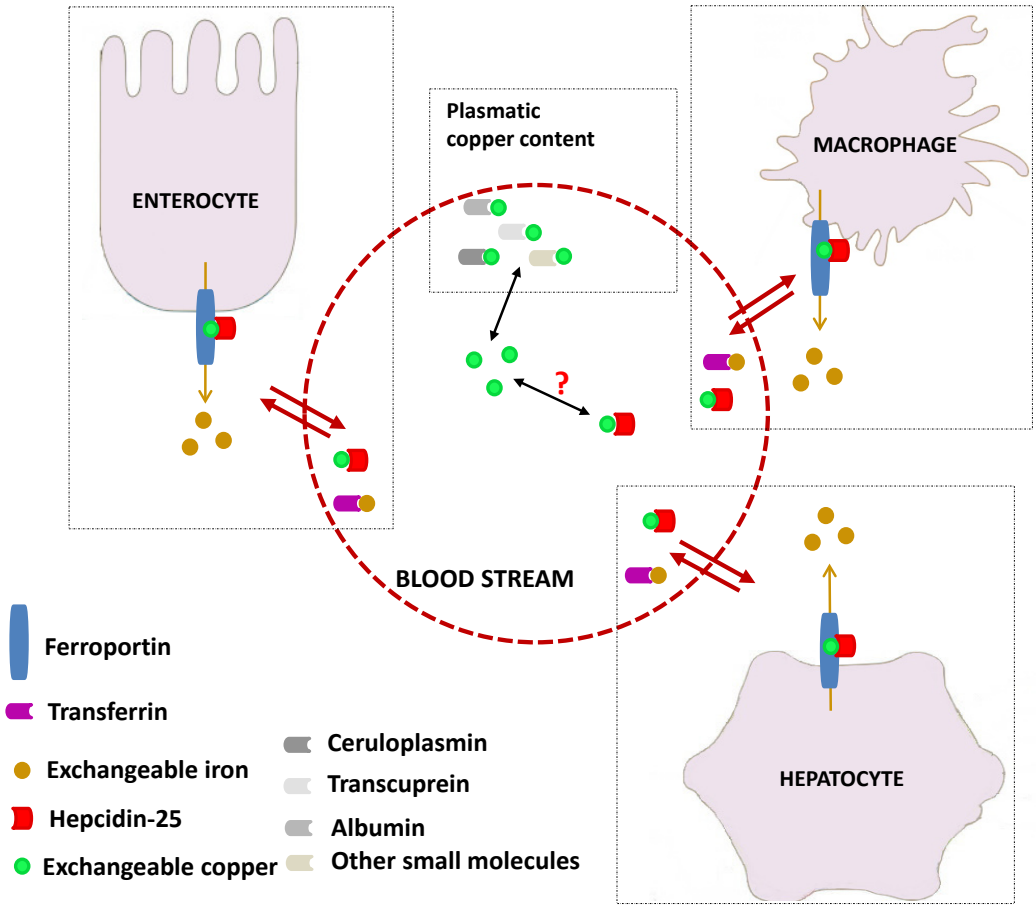

Figure 3.13: Iron homeostasis. Suggested iron and copper traffic in the blood stream. (Ferroportin (blue) is expressed on the membrane surface of iron releasing cells (enterocytes, hepatocytes and macrophages), allowing the iron ions (yellow) to exit the cell (yellow arrow) and bind transferrin (pink) for transport through the blood stream. Circulating hepcidin-25 (red) blocks the iron export by binding to ferroportin. Copper ions (green) released from intestinal cells are bound by various molecules in blood. Iron and copper ions are exchanged between various complexing agents based on chemical affinities and biological needs).

Most of the copper present in humans is bound to ceruloplasmin, albumin and transcuprein, however, the copper trafficking in the human body is not fully elucidated. Small amounts of copper can be exchanged to low molecular weight molecules (see *section 1.2.3.3.*). Considering the high affinity of the 6-residue N-terminus of hepcidin-25 for copper ($K_D=10^{-14.66}$ M) [112], the concentration of "free" extracellular Cu^{2+} available to small molecules and peptides of 1-2 µM [114] (see *section 1.2.3.3.*) and the mean physiological level of hepcidin-25 in humans of 5-15 µg/L (1.8-5.4 nM) (see *section 1.3.3.3.*), it is reasonable to assume that a considerable fraction of hepcidin-25 is present in the copper-bound form under physiological conditions (Figure 3.13).

Additionally, a screening of hepcidin sequences in the UniProt (Universal Protein resource) databank showed that the 6-amino acid N-terminus of hepcidin-25 containing the ATCUN motif is conserved among most of the species (Table 3.3), which suggests a specific receptor interaction.

Table 3.3: Conservation of hepcidin-25 amino acid (AA) sequences among some species (blue-conserved AA in the 6-residue N-terminus, red-different AA in the 6-residue N-terminus).

Species	Uniprot* code	Hepcidin-25 AA sequence
Human	P81172	**DTHFPI**CIFCCGCCHRSKCGMCCKT
Bovine	Q2NKT0	**DTHFPI**CIFCCGCCRKGTCGMCCRT
Rabbit	G1U3P2	**DTHFPI**CIFCCSCCRNSKCGICCKT
Dog	Q5U9D2	**DTHFPI**CIFCCGCCKTPKCGLCCKT
Sheep	E0X9N1	**DTHFPI**CIFCCGCCRKGTCGICCKT
Pig	Q8MJ80	**DTHFPI**CIFCCGCCRKAICGMCCKT
Chimpanzee	A7XEH6	**DTHFPI**CIFCCGCCHRSKCGMCCKT
Sumatran orangutan	Q5NVR8	**DTHFPI**YIFCCGCCHRSKCGMCCKT
Mouse	Q9EQ21	**DTNFPI**CIFCCKCCNNSQCGICCKT
Rat	Q99MH3	**DTNFPI**CLFCCKCCKNSSCGLCCIT
Crocodile	E8ZAD0	**NSHFPI**CSYCCNCCRNKGCGLCCRT

* Universal Protein resource [218]

Hence, analytical techniques may be needed for the analysis of the copper-bound form of hepcidin-25. Investigations of the ATCUN-copper(II) complex within hepcidin-25 were performed using UV-Vis spectroscopy, LC-MS/MS (Triple Quadrupole) and FTICR-MS, and will be presented in the following sections.

3.2.1. Peptide DTHFPI-copper(II) complex

To study the formed metal complex between hepcidin-25 and copper, first, the 6-amino acid N-terminus containing the ATCUN motif was considered (Figure 3.14). The N-terminus of Hep-25 was reported to be unstructured, in contrast to the rest of the molecule, which is highly compact due to presence of four intramolecular disulfide bridges [85] (see *section 1.2.2.*). This indicated that the flexible NH_2-terminus is a good model for hepcidin-25 structural analysis. Additionally, the use of the N-terminus was previously reported in bioactivity studies of hepcidin-25 [116] (see *section 1.2.4.1.*) and in ATCUN-copper(II) interaction studies [97, 112] (see *section 1.2.3.3.*).

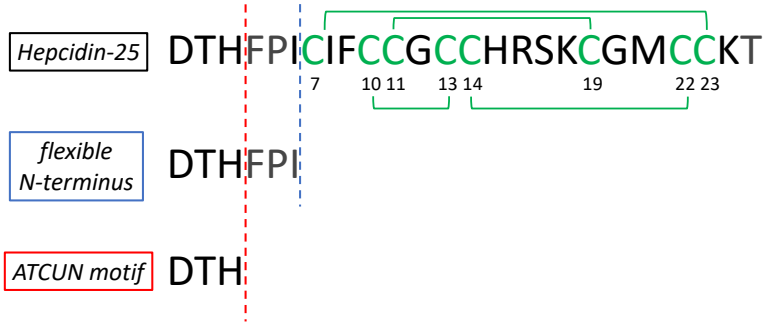

Figure 3.14: Amino acid sequence of hepcidin-25, its 6-residue N-terminus and AT-CUN motif.

The behavior of the 6-amino acid N-terminus (DTHFPI) was investigated spectrophotometrically in the presence of copper ions at physiological pH (7.4). Initially, a 1 mM peptide solution was tested in the presence of an equimolar Cu(II) solution. However, such low concentrations did not lead to significant changes detected in the optical spectrum of the peptide at 525 nm. Further, a 10 mM peptide solution was titrated with 10 mM copper (Cu^{2+}) solution. The binding of copper ions to the peptide DTHFPI yielded an absorption maximum at 525 nm, which is an indicative of the ATCUN motif [100]. In the case of peptides and proteins that do not coordinate copper, a broad maximum near 600 nm is observed, which eventually shifts to 525 nm only at pH > 10 [97].

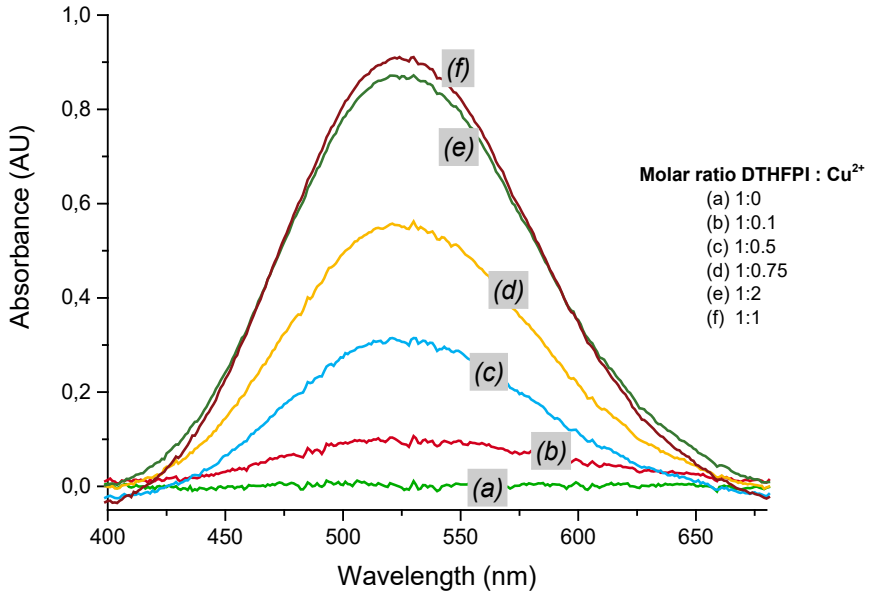

Figure 3.15: Optical spectra of 10 mM Cu^{2+} binding to 10 mM peptide DTHFPI (HEPES, pH 7.4) at different molar ratios.

As it can be seen in Figure 3.15, the copper complex was formed even at a molar ratio peptide:Cu^{2+} of 1:0.1. The signal intensity increased as the molar ratio peptide:Cu^{2+} increased. A saturation was reached at the molar ratio of 1:1. The complex was also found stable in the presence of copper ions in excess (molar ratio 1:2). Such large concentrations (10 mM) would imply high UV-Vis experimental costs in the case of full hepcidin-25. Additionally, the "sticky" character of the peptide (see *section 3.3.2.1.*) could hinder its robust handling in plastic and quartz cuvette. Therefore, MS-based techniques were employed for hepcidin-25-copper(II) complex analysis.

3.2.2. Chromatographic separation of hepcidin-25-copper(II) complexes

The MS-based analysis of the hepcidin-25-copper(II) complex using FTICR or MALDI-MS has so far been performed without the separation of the copper-bound and copper-free peptide (see *section 1.2.3.2.*). This hampers an accurate and sensitive analysis of a mixture of the peptide species, especially in more complex matrixes such as biological samples. The addition of an LC separation step in the MS analysis improves analyte detection and reduces ion suppression effect. Anion exchange chromatography has been previously employed for the separation of the copper-labeled peptide from the excess of copper in an LC-ICP-MS method, which was developed for the quantification of Hep-25 by measuring the amount of copper bound to the peptide [108] (see *section 1.3.2.1.*). Unfortunately, this method provides no molecular information of the peptide species present in the solution. Also, the limited stability of the anion exchange columns complicates a robust and reproducible analysis.

In the present work, a RP-HPLC (reversed phase high performance liquid chromatography) separation step, which was complementary to MS detection, was tested for an accurate and robust analysis of the species resulting from the complexation of Hep-25 with copper. Previous reports showed that the complex hepcidin-25-copper(II) is not stable at pH <5.2 (see *section 1.2.3.3.*). This can be explained by the fact that, in order to bind to the biomolecule, the metal ion must replace hydrogen ions of the peptide, which is facilitated by increasing the pH of the peptide solution. Thus, for LC analysis of the copper-bound form of hepcidin-25, a neutral to basic pH of the mobile phases was required for metal complex stability. Initially, a chromatographic separation at physiological pH (7.4) was tested (data not shown). This proved to be unsuccessful, probably because a good chromatographic resolution is usually achieved when fully ionized or fully non-ionized species are examined. Hepcidin-25 is a basic peptide with the isoelectric point of 8.2. More than 99% of hepcidin will be ionized at a pH value approximately 2 pH units above its isoelectric point of 8.2. However, such high pH values are usually not suitable for commonly used conventional reversed phase chromatography columns, stable up to a pH of 8. Nevertheless, in the present work new generation HPLC columns were employed for performance at high pH. An ACE Super Phenyl-Hexyl HPLC column with extended pH stability allowed the HPLC analysis at pH 11, using mobile phases containing 0.1% ammonia (chromatographic set 4, see *section 2.4.2.2.*). The formation of the hepcidin-25-copper(II) complex at different molar ratios and at various pH values was tested and will be discussed in the following sections.

3.2.2.1. Influence of molar ratio on hepcidin-25-copper(II) complex behavior

The simultaneous monitoring of the metal complex and the remaining free hepcidin-25 was performed via LC-MS analysis by addition of a 1.8-180 µM Cu^{2+} solution to an 18 µM (50 mg/L) hepcidin-25 solution in a 0.1-10 molar excess (see *section 2.2.6.*). The complexation of the peptide with the copper(II) ions was performed at a pH value of 11 (as the pH of the employed mobile phases). Three peaks were identified (Figure 3.16) and subsequently attributed

(via MS/MS) to hepcidin-25 (copper-free) and the complex of hepcidin-25 with one and two copper ions respectively.

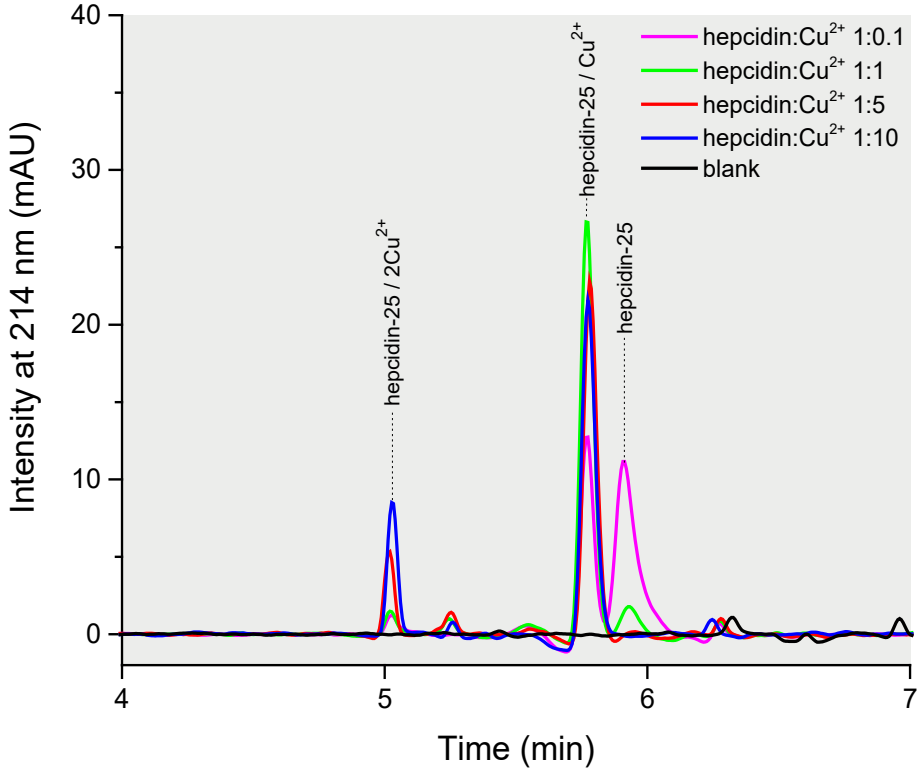

Figure 3.16: The influence of molar ratio on hepcidin-25-copper(II) complex behavior at pH 11 (HPLC separation) (mobile phase A: H_2O/NH_3 100/0.1 v/v pH 11, B: $ACN/H_2O/NH_3$ 90/10/0.1, hepcidin-25 concentration 50 mg/L (18 µM)).

The HPLC peaks were recorded in the wavelength range of 210-220 nm, where absorbance arises primarily due to peptide bonds. No signal was detected at 525 nm (absorption maximum of ATCUN-Cu(II) complex), probably due to lower concentrations of the analyte (18 µM). Copper has a smaller value of the extinction coefficient in comparison to peptides, thus higher concentrations are needed for DAD detection. This came in accordance with previous UV-Vis experiments (see *section 3.2.1.*), where a concentration of 10 mM peptide-copper(II) led to UV-Vis signal at 525 nm.

As expected, the DAD signal at 214 nm showed a decrease in the hepcidin amount with the addition of Cu^{2+} which could be explained by the metal complex formation (Figure 3.16). Interestingly, a significant amount of copper complex was formed even at a molar ratio hepcidin-25:Cu^{2+} of 1:0.1, in agreement with previously described results involving the 6-residue N-terminus DTHFPI (see *section 3.2.1.*). Additionally, the decrease in hepcidin-25 signal at molar ratio 1:5 and 1:10 coincides with significantly increased amounts of a second compound identified by MS/MS as the complex of hepcidin with two copper ions. This species was briefly mentioned previously in the literature [109], but no comprehensive MS characterization of the

complex was performed. Figure 3.16 also shows that at pH 11 there was unreacted peptide present at an equimolar ratio, while the excess of copper (1:5) led to quantitative complex formation. Contrarily, in the case of the 6-amino acid N-terminus, the saturation was achieved at a molar ratio of 1:1. However, the experiments with the short peptide were performed at physiological pH (7.4). At this point it was concluded that the behavior of hepcidin-25-copper(II) complex is pH-dependent. This is also supported by previous reports, which show different results for quantitative hepcidin-25-copper(II) complex formation at different pH values. Some authors applied FTICR-MS measurements at pH 6.8 and found total complex formation at a molar ratio hepcidin-25:copper of 1:1 [107], while others recorded the presence of unreacted peptide at equimolar concentrations, in a q-TOF-MS (quadrupole-time-of-flight mass spectrometry) experiment performed at pH 8.2 [108]. In the case of the applied pH of 11, an explanation for the presence of free peptide could be that the high pH determined by 0.1% NH_3 drives the copper ions to form highly stable complexes with the hydroxyl and ammonia groups. In the light of these reports, the influence of the pH value of the peptide-copper(II) solution was investigated further.

3.2.2.2. Influence of pH on hepcidin-25-copper(II) complex behavior

The behavior of the metal complex prepared in different buffers (pH 2.2, 4.2, 7.4 and 11 respectively) was tested using stoichiometric Cu^{2+} solution (molar ratio 1:1). The chromatographic separation was achieved in all cases using mobile phases containing 0.1% ammonia. The results confirmed complete complex formation of hepcidin-25 with one copper ion at physiological pH. However, at basic pH (11), traces of free peptide could be observed (Figure 3.17), as described in the previous section. Surprisingly, the species containing one copper ion was present even at pH values below the reported complex stability limit of 5.2, namely 4.2 and 2.2. This could be explained by rapid metal complex formation during sample injection, promoted by the basic pH of the LC mobile phases (11). Additionally, the formation of the peptide complex with two copper ions occurred preferably at high pH (11). At physiological pH (7.4), the metal complex present was mainly the mono-copper(II) complex. These findings were in line with the previously described results at a pH value of 6.8.

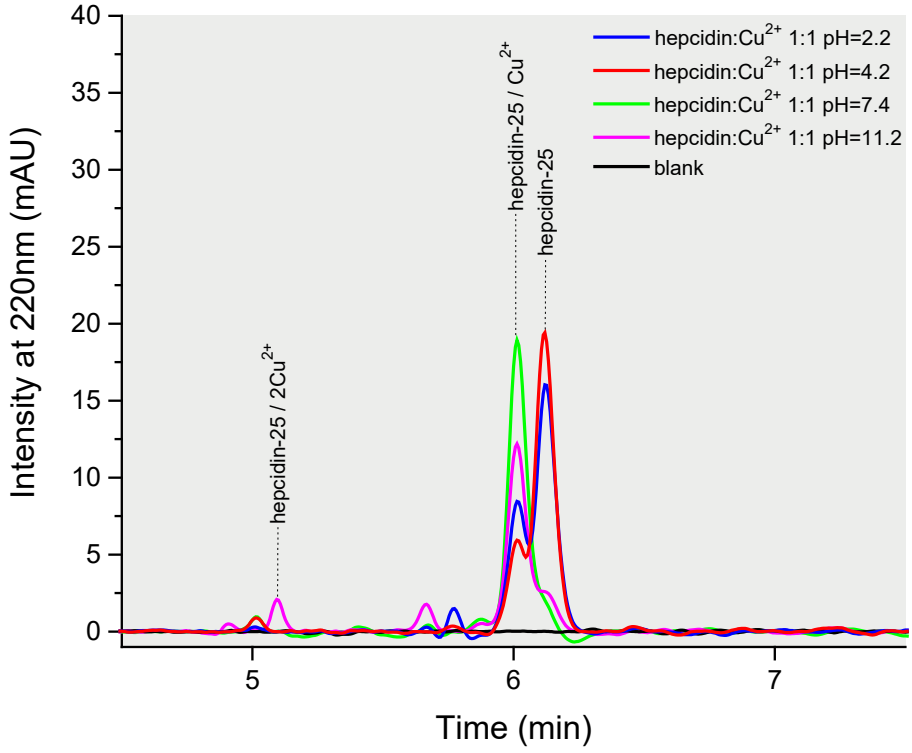

Figure 3.17: The influence of pH on hepcidin-25-copper(II) complex behavior at molar ratio 1:1 (HPLC separation) (mobile phase A: H_2O/NH_3 100/0.1 v/v pH 11, B: $ACN/H_2O/NH_3$ 90/10/0.1, hepcidin-25 concentration 50 mg/L (18 µM)).

3.2.3. LC-MS/MS analysis of hepcidin-25-copper(II) complex(es) at pH 11

Mass spectrometric detection of hepcidin-25 and hepcidin-25-copper(II) was performed using a triple quadrupole (QqQ-MS), coupled to the HPLC. Previously presented chromatographic separation (chromatographic set 4, see *section 2.4.2.2.*) by employing mobile phases at pH value of 11 was used. The samples containing a peptide solution of 50 mg/L (18 µM), prepared in 0.1% ammonia at pH 11 (the solvent mixture at the start of the HPLC gradient), were analyzed at different molar ratios of hepcidin-25:Cu^{2+} (1:1, 1:10). ESI-MS parameters such as curtain gas flow, nebulizer gas or ion spray voltage were optimized. Full scan mode (Q1+) and product ion mode (MS2+) were applied. Collision induced dissociation (CID) was employed for fragmentation purposes. Initially, ESI was operated in negative mode (ESI-), which resulted in poor specific quasi-molecular ions and no specific fragmentation of the most abundant quasi-molecular ion ($[M+3H]^{3+}$) could be achieved. Therefore, positive mode (ESI+) was employed further.

Hepcidin-25

First, a solution of hepcidin-25 (50 mg/L) was tested. Full scan mode (+Q1) was employed in the range of m/z ratio between 100 and 2000. The doubly, triply and quadruply charged quasi-molecular ions were identified with m/z of 1394.6 ($[M+2H]^{2+}$), 930.3 ($[M+3H]^{3+}$) and 697.8

([M+4H]$^{4+}$) respectively. [M+3H]$^{3+}$ was the most abundant quasi-molecular ion and was selected as precursor ion for fragmentation. Product ion spectra (+MS2) resulted in the loss of b_3 (354 Da) and b_4 (500.4 Da) to yield y_{22}^{2+} (1218.2 Da) and y_{21}^{2+} (1144.7 Da) respectively (Figure 3.18).

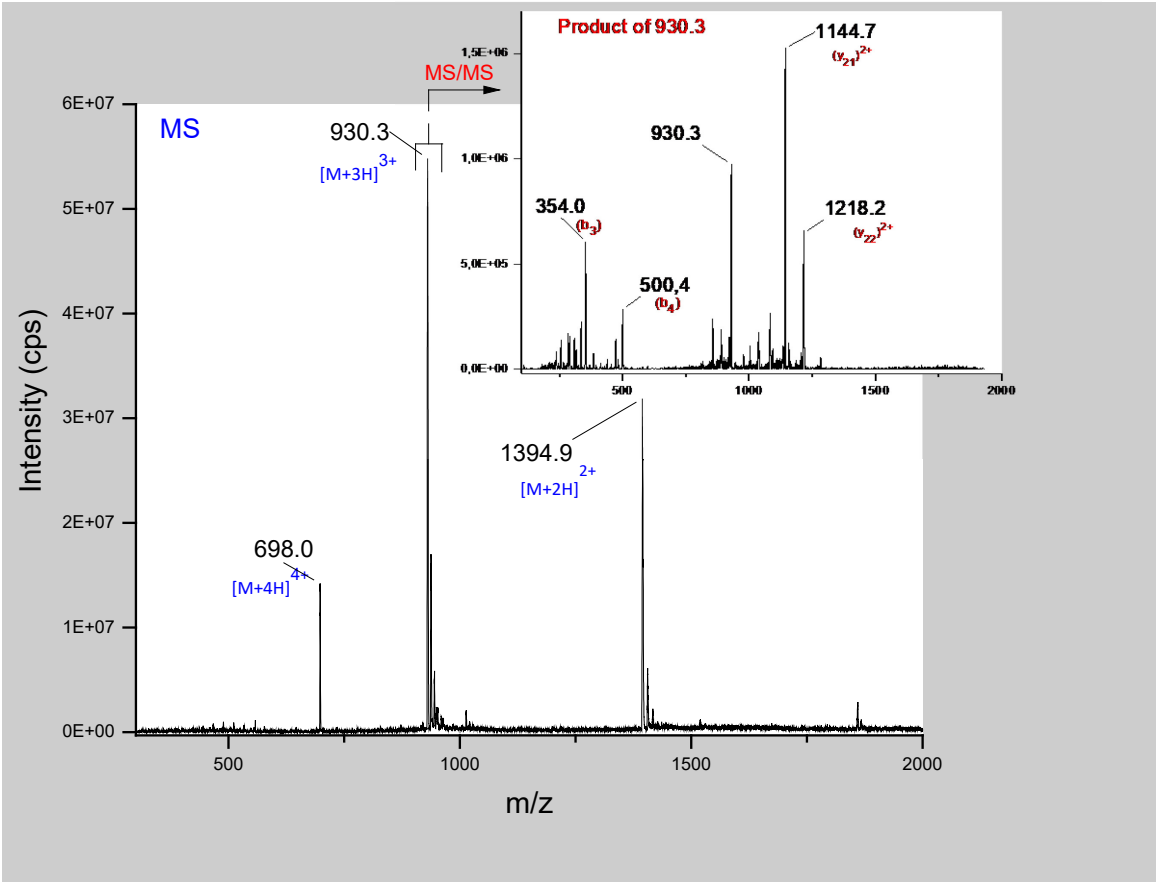

Figure 3.18: Full scan (MS) and product ion (MS/MS) spectra of hepcidin-25 (basic mobile phase, pH 11).

The fragmentation of hepcidin-25 (Figure 3.19) seems to be highly influenced by the intramolecular disulfide bonds and the ATCUN motif. N-truncation occurred before the first cysteine in position 7. This yielded 3- or 4-amino acid fragments, the shortest of which corresponded to the metal binding motif ATCUN (DTH), illustrating its stability during fragmentation. C-fragmentation resulted in longer fragments encompassing all four disulfide bridges. This proved the stability of disulfide network.

The calculated mass of hepcidin-25 resulting from the triply charged quasi-molecular ion was found to be 2787.9 Da. The difference of roughly 0.9 Da as compared to the theoretical exact mass of 2787.0258 Da could be explained by taking into consideration the uncertainty of the unit mass resolution (considered Full Width at Half Maximum FWHM) of the triple quadrupole MS device. Accurate masses were confirmed by employing HR-MS (see *section 3.2.4.*).

Figure 3.19: Fragmentation pattern of hepcidin-25 in ESI MS.

Hepcidin-25-Cu^{2+}

Further, the analysis of the hepcidin-Cu^{2+} solution (molar ratio 1:1) showed similar series of ions in full scan mode (+Q1) as compared to hepcidin-25. The quasi-molecular ions with m/z of 1425.0 ([M+Cu]$^{2+}$), 950.3 ([M+H+Cu]$^{3+}$) and 713.1 ([M+2H+Cu]$^{4+}$) were identified (Figure 3.20).

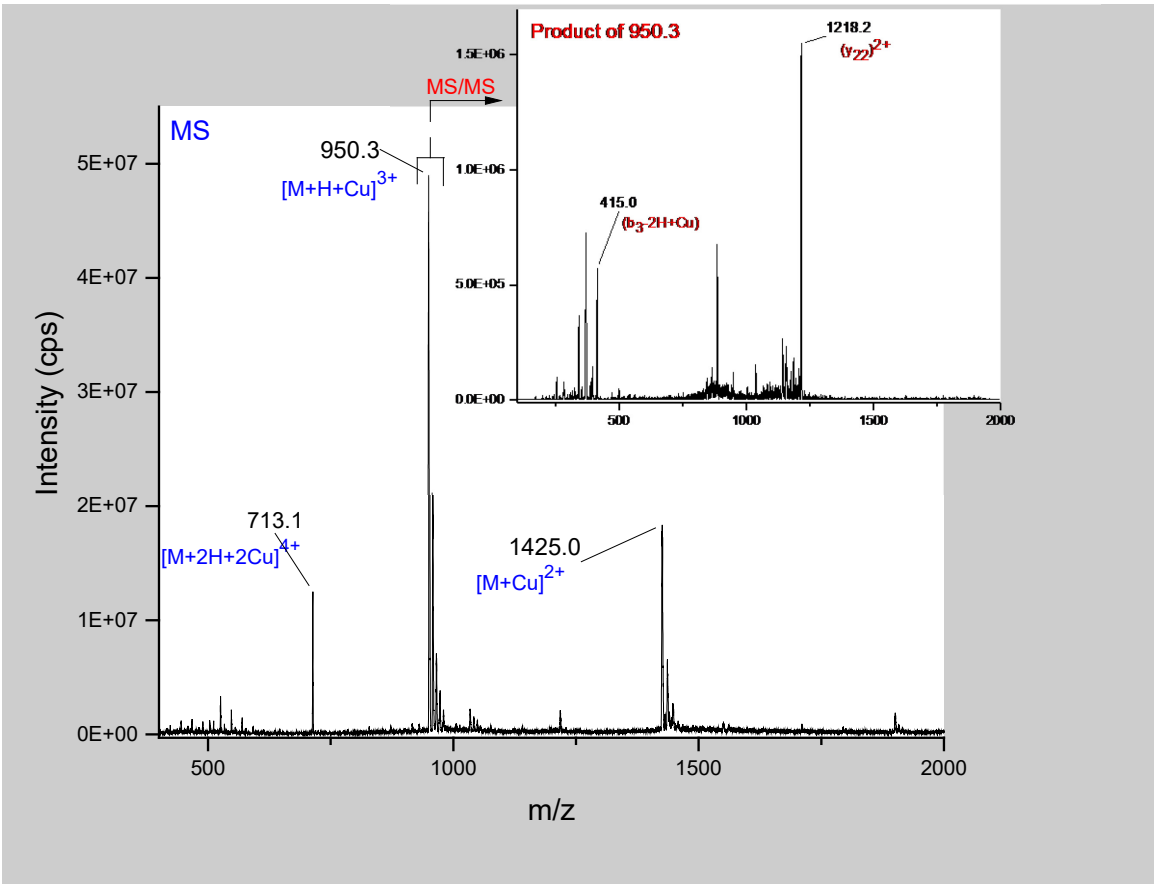

Figure 3.20: Full scan (MS) and product ion spectra (MS/MS) of hepcidin-25-Cu^{2+} (basic mobile phases, pH 11).

The fragmentation of the triply charged quasi-molecular ion as the most intense precursor ion resulted in the same fragment y_{22}^{2+} (1218.2 Da) as in case of hepcidin-25. Another fragment (415 Da) was attributed to the copper-adduct of b_3, which represents the N-terminal fragment containing the three amino acids at the ATCUN motif (DTH). The difference of 61 Da corresponds indeed to the addition of one copper (62.9 Da), with the loss of 2 Da and 2 positive charges (2 H$^+$), in accordance to previous findings [108, 107]. The species could be identified as hepcidin-25 complexed with one copper ion binding the ATCUN motif. The fragmentation

pattern of the peptide complexed with one copper ion showed similar behavior as compared to hepcidin-25. The compact structure determined by the four intra-molecular disulfide bridges was preserved in the product ion mode. Also, the ATCUN motif complexed with Cu^{2+} remained intact during fragmentation, demonstrating the stability of the metal complex. This is in accordance to previously reported findings [108, 107]. The calculated mass of hepcidin-25-Cu^{2+} complex was determined to be 2847.9 Da, which is comparable to the theoretical exact mass of 2847.9397 Da. The accurate mass and the isotope pattern corresponding to a copper-containing peptide were confirmed by HR-MS as in the case of hepcidin-25 (see *section 3.2.4.*).

$2Cu^{2+}$/Hepcidin-25

The analysis of the solution of hepcidin-Cu^{2+} (molar ratio 1:10) revealed a two Cu^{2+}/hepcidin-25 species. The peptide complexed with two copper ions was previously reported in literature using MALDI-TOF analysis [109], but never characterized using LC-MS/MS.

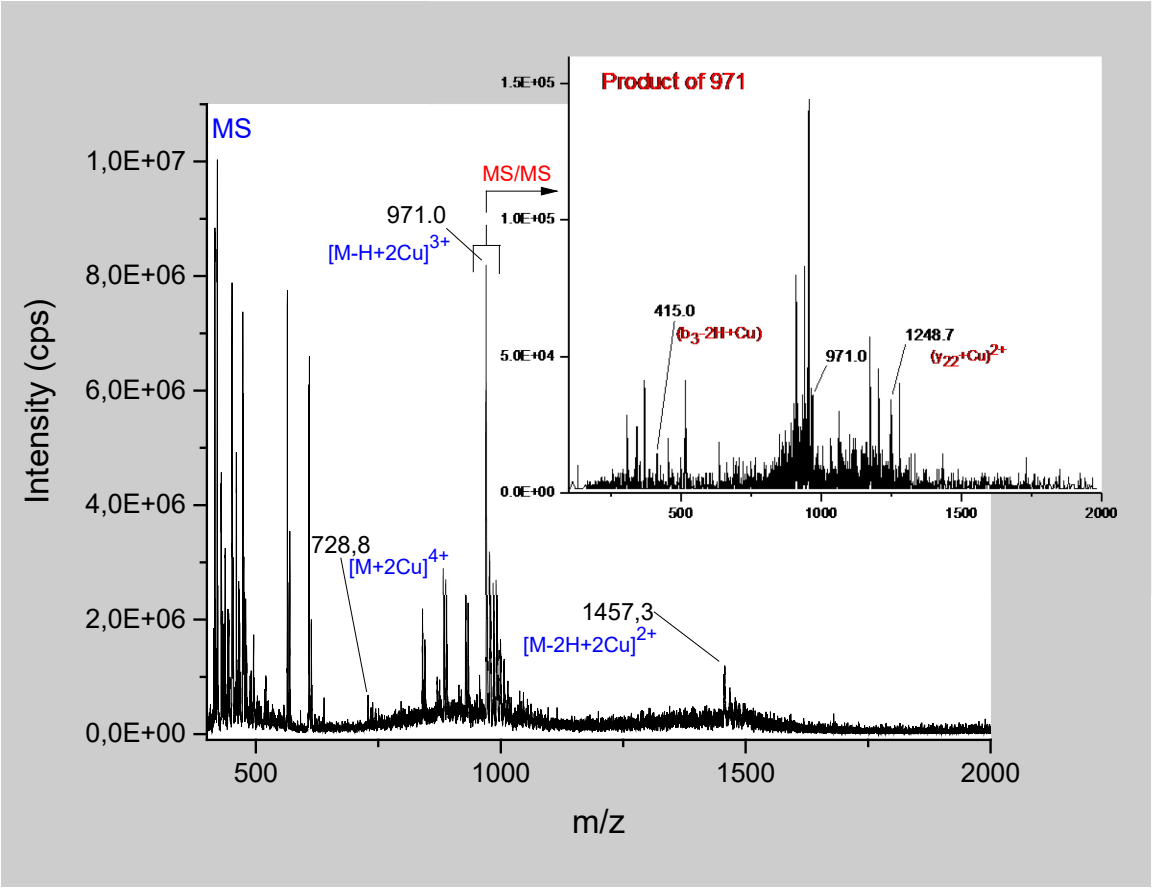

Figure 3.21: Full scan (MS) and product ion spectra (MS/MS) of hepcidin-25 complexed with two Cu ions (basic mobile phases, pH 11).

The doubly, triply and quadruply charged quasi-molecular ions were observed with m/z ratio of 1456.1, 971.0 and 728.5 respectively (calculated mass 2910 Da), but the signal intensity was lower compared to the other two species (Figure 3.21), probably due to a lower concentration, which was also observable in the LC chromatogram.

The product ion spectra of the triply charged quasi-molecular ion allowed the identification of the 415 Da fragment corresponding to the copper-adduct of the DTH motif. Another fragment of 1248.7 Da was identified that could be attributed to a copper-adduct of the fragment $(y_{22})^{2+}$, taking into consideration the mass difference of 30.5 Da for the doubly charged fragment. This indicated a two Cu^{2+}/hepcidin-25 species with one copper ion complexed at the metal binding motif and a second Cu^{2+} binding the rest of the biomolecule. This fact has never been reported before and it could be speculated that the second copper ion binds the histidine in position 15, due to the high affinity of the imidazole group for copper. Further investigations of the interaction of hepcidin-25 to the second copper ion were performed using HR-MS (see *section 3.2.4.*). Hepcidin-25 was analyzed on a triple quadrupole in positive mode. Remarkably, even at pH 11, the ESI ionization process worked efficiently in positive mode leading to a sensitive detection of protonated hepcidin species. This shows that the ionization process is not always correlated to the pH of the sample, which is understandable, when considering the complexity of the ion formation process in gas phase.

3.2.4. HR-MS of hepcidin-25-copper(II) complex

The triple quadrupole coupled to HPLC offers excellent sensitivity for quantification of biomolecules. However, the limited mass resolution does not permit reliable structural characterization of peptides or metal-peptide complexes (accurate mass, isotope composition determinations). Thus, in order to validate the results obtained at unit-mass resolution and to accurately characterize the isotope pattern of hepcidin-25 and its complexes with copper, high resolution MS (HR-MS) was employed. For this purpose, FTICR-MS experiments were performed by operating the mass spectrometer in positive mode (ESI+) and at highest resolution (FWHM 10^6). Direct infusion of the hepcidin-25-copper(II) solution prepared at a pH of 11 (see *section 2.2.6.2.*) resulted in the identification of the two peptide-copper(II) species. The triply charged quasi-molecular ions presented isotope patterns corresponding to the chemical formula $C_{113}H_{168}N_{34}O_{31}S_9Cu$ (Figure 3.22) and $C_{113}H_{168}N_{34}O_{31}S_9Cu_2$ respectively (Figure 3.23). A mass accuracy in the range of 1-4 ppm was obtained using the formula "mass accuracy (ppm) = (accurate mass – exact mass)/exact mass $\cdot 10^6$" [213].

The mass difference between hepcidin-25 and its complexes with one and two copper ions was 60.9 Da (63.9-2) and 124.8 (2·63.9-2) Da respectively. This indicated that the interaction with the first copper ion occurred with the loss of two H^+ as confirmed previously by LC-MS/MS. This correlated with the formation of the ATCUN-copper(II) complex which generally takes place with deprotonation of the two nitrogen atoms involved in peptide bonds. However, the second copper ion binds without proton expulsion. This could represent a much weaker interaction of copper(II) with the second histidine residue in position 15. The extra nitrogen donor atom of arginine residue, found in the immediate vicinity of histidine, or the alcoholic-OH of serine in position 17 could contribute to the coordination chemistry which might characterize this interaction [219].

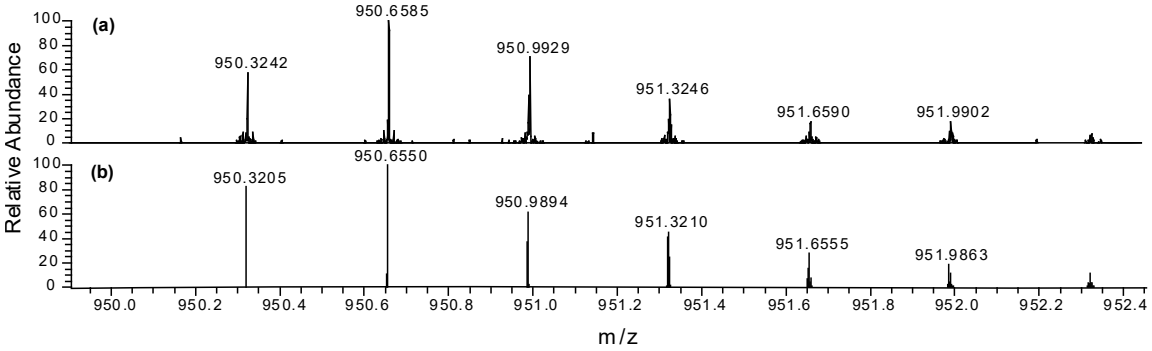

Figure 3.22: FTICR-MS spectra of hepcidin-25/Cu^{2+} at pH 11 ($C_{113}H_{168}N_{34}O_{31}S_9Cu_1$ + 3H, charge +3): (a) experimental accurate mass; (b) theoretical exact mass.

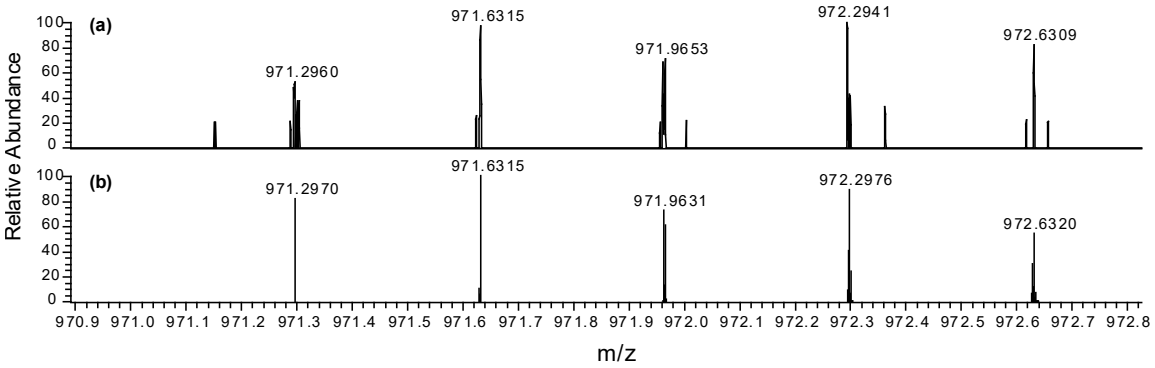

Figure 3.23: FTICR-MS spectra of hepcidin-25/2Cu^{2+} at pH 11 ($C_{113}H_{168}N_{34}O_{31}S_9Cu_2$ + 3H, charge +3): (a) experimental accurate mass; (b) theoretical exact mass.

The FTICR-MS experiments were performed in the absence of LC mobile phases (pH 11), using direct infusion. This permitted, additionally as in the case of LC-MS/MS experiments, the comparison between the behavior of hepcidin-25/Cu^{2+} at basic and at physiological pH. Very similar isotope patterns of hepcidin/Cu^{2+} were obtained at both pH values of 7.4 (Figure 3.24) and 11.

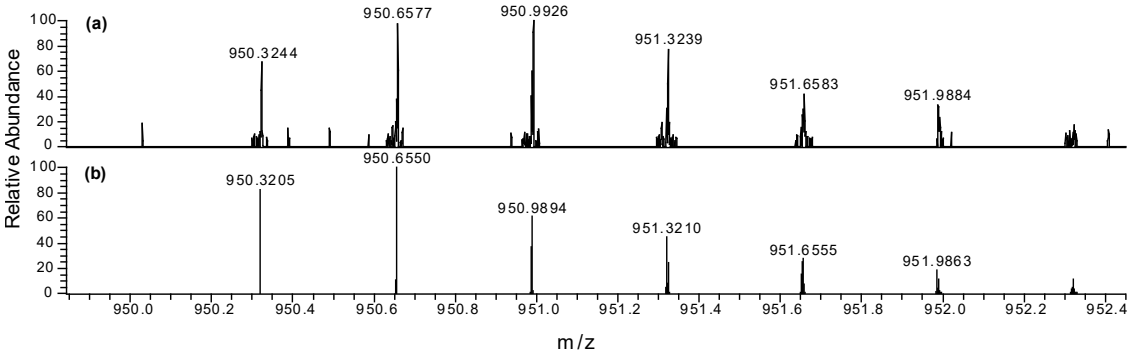

Figure 3.24: FTICR-MS spectra of hepcidin-25-Cu(II) at pH 7.4 ($C_{113}H_{168}N_{34}O_{31}S_9Cu_1$ + 3H, charge +3): (a) experimental accurate mass; (b) theoretical exact mass.

FTICR offered superior resolution compared to the triple quadrupole, which has been used previously in the LC-MS/MS experiments. However, HR-MS would be less attractive for quantification purposes. In this regard, the developed chromatographic separation at basic pH com-

plemented by MS/MS detection using the triple quadrupole could represent in future a quantification tool of the copper-bound hepcidin-25 form. This would require however the development of a sample preparation protocol at physiological or basic pH which would assure metal complex stability.

3.2.5. 3D-model structure of hepcidin-25-copper(II)[1]

In the light of the mass spectrometric characterization of hepcidin-25/Cu^{2+}, further structural analysis of the metal complex employing NMR was performed, with the development of a first structural model of the Cu^{2+}-bound hepcidin-25.

Initially, the solution structure of the N-terminal hexapeptide complexing Ni^{2+} at physiological pH (7.4) was determined based on NMR experimental data. The strong paramagnetism of the Cu^{2+} ATCUN complex gives rise to large chemical shift changes and line broadening effects of the NMR resonances preventing high structural resolution close the metal ion [101]. Thus, Ni(II) ions were employed that also bound Hep-25 with high affinity and serve an appropriate substitute for Cu^{2+} forming a diamagnetic ATCUN complex amenable to high-resolution structure determination by NMR [220, 103]. Internuclear distance restraints were calculated from ROESY cross peak volumes, and dihedral angle restraints of the peptide backbone were generated from backbone chemical shifts using the program TALOS. In addition, the square-planar coordination known to be present in metal-bound ATCUN motifs (see *section 1.2.3.1.*) was imposed by additional distance and dihedral angle restraints.

Subsequently, models of full-length hepcidin complexing Cu^{2+} were generated by combining the 3D structure of the N-terminal hexapeptide, described above, with the 3D structure of the 19 C-terminal amino acids as determined by Jordan *et al.* (PDB code: 2KEF) [85]. The resulting model with lowest restraint violation energy of copper-bound hepcidin-25 is shown in Figure 3.25 [221].

[1] The work presented in this section was conducted by Marija Vranic and Heiko Möller at the University of Potsdam.

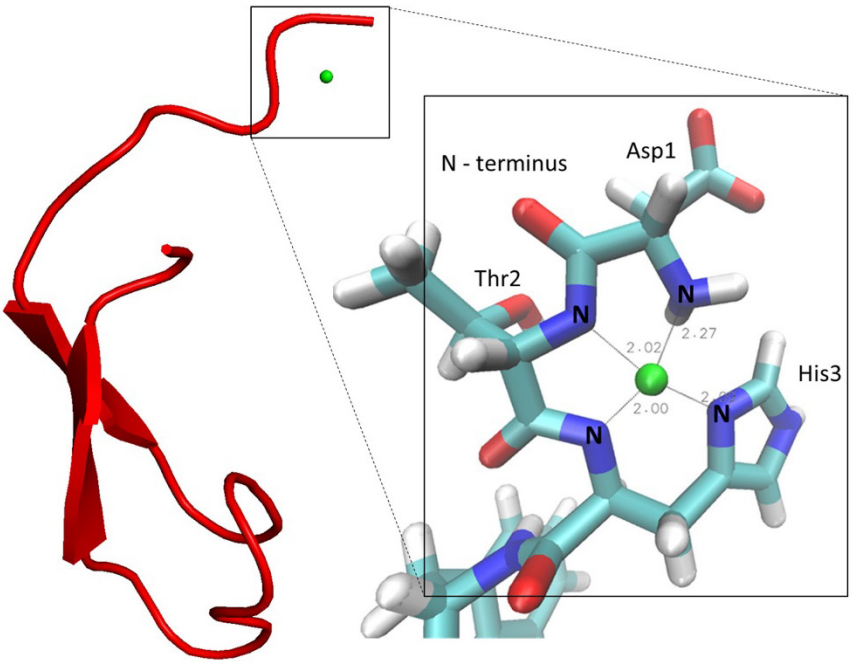

Figure 3.25: The lowest energy structure of the hepcidin 25/Cu^{2+} model structure (Figure provided by Marija Vranic and Heiko Möller, University of Potsdam, Germany).

The results of the LC-MS/MS, HR-MS and NMR investigations of hepcidin-25/Cu^{2+} support the theory that copper is as an inherently incorporated entity in the native form of the iron regulator. This copper-peptide could offer more relevant information regarding iron homeostasis as compared to hepcidin-25. However, further research should be conducted in this direction to identify hepcidin-25/Cu^{2+} in biological samples and investigate its biological function.

3.3. LC-MS/MS methods for the quantification of hepcidin-25 in human serum

Sections 3.1. and 3.2. mainly comprised qualitative studies of the peptide hepcidin-25. High concentration ranges (50-250 mg/L), which occurred in the course of the folding process, solubility investigations or during the studies regarding the formation of the peptide-copper complex, allowed the use of HPLC for peptide amount determination. Mass spectrometry or tandem mass spectrometry were complementarily used for mass characterization and identity confirmation of hepcidin-25 species. However, for the development of quantification methods suitable for the assessment of Hep-25 in biological samples (human serum), improved sensitivity is required. In healthy individuals, the concentration of hepcidin-25 varies from below reported LOQ of around 1 µg/L to 25-40 µg/L (see *section 1.3.3.3.*). Therefore, HPLC and MS/MS were coupled to develop a selective and sensitive analytical method for peptide quantification. Isotope-labeled hepcidin-25 was employed as internal standard (IS). Based on the median and mean physiological level defined previously at 1-5 µg/L and 5-15 µg/L respectively (see *section 1.3.3.3.*), the targeted dynamic range for Hep-25 quantification in healthy subjects was considered to be between 0.5 µg/L and 40 µg/L.

As discussed in *section 1.3.2.3*, isotope dilution liquid chromatography tandem mass spectrometry ID-LC-MS/MS (particularly using triple quadrupole) is increasingly used as an alternative to immunoassays in the field of clinical chemistry and laboratory medicine for quantification purposes. Moreover, several internationally well reputed institutions such as CLSI or NIST acknowledge (ID-)LC-MS/MS as the "gold standard" for small biomolecule quantification [168, 5].

3.3.1. LC-MS/MS work flow

The development of a LC-MS/MS method for quantification of Hep-25 in human serum implied 3 major steps which are typical for clinical determination of endogenous biomolecules in biological samples (Figure 3.26):

- Sample preparation
- Chromatographic separation (HPLC)
- Detection by tandem mass spectrometry (MS/MS)

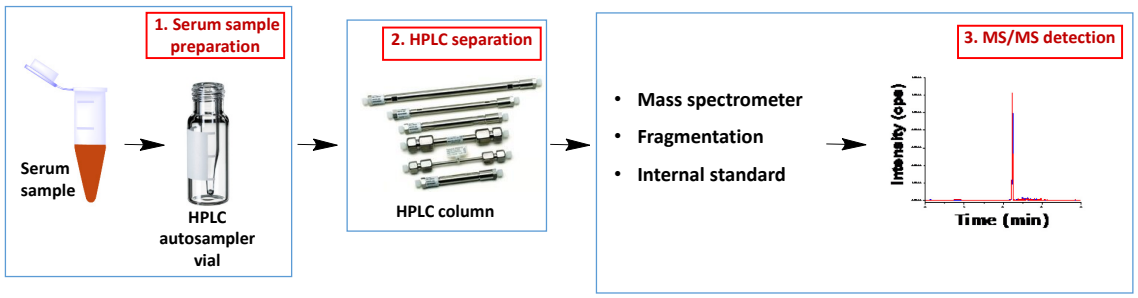

Figure 3.26: Work flow for Hepcidin-25 quantification emplyoing LC-MS/MS [222].

In the present study, two sample preparation protocols were developed for hepcidin-25 extraction (see *section 3.3.2.3.*) and two chromatographic separation strategies were tested by employing acidic, TFA-based, (see *section 3.3.3.1.*) and basic, ammonia-containing, mobile phases (see *section 3.3.3.2.*). A triple quadrupole instrument operated in MRM mode was used for peptide quantification (see *section 1.3.2.3.*). The final goal was the development and validation of a LC-MS/MS quantification method for hepcidin-25 that can be recommended for routine laboratory implementation (see *section 3.4.*). By such, in addition to good precision, accuracy and sensitivity, the method required characteristics such as fast, robust and cost-effective, implying simple procedures, economic reagents involved and low sample volume used. Therefore, each step of the LC-MS/MS method was selected in accordance with these aspects. Additionally, the properties of the desired analyte, Hep-25, were taken into consideration. In this regard, its amino acid content (one acidic amino acid and five basic residues as presented in *section 3.1.*) could strongly influence the LC-MS/MS analysis. The choice of the sample preparation technique, the chromatographic protocol (HPLC column and other parameters) and

the ionization parameters depend on the charge of the molecule and the hydrophilicity/hydrophobicity ratio. Moreover, the reported "stickiness" of hepcidin-25 [84, 93] was investigated to determine its influence on the LC-MS/MS analysis.

3.3.2. Sample preparation

3.3.2.1. Hepcidin-25 adsorption

Due to their generally amphipathic character, peptides and proteins readily adsorb to most surfaces such as glass or plastic laboratory ware. When LC-MS quantification of a peptide is the main end-point, the adsorption effects need to be evaluated [201]. In the case of hepcidin-25, the "stickiness" of the peptide is well known from literature [84, 93, 55]. Since the LC-MS/MS quantification can be severely affected by a non-specific interaction of the peptide to the laboratory ware in terms of reproducibility, sensitivity and accuracy, the adsorption effects were tested prior to further LC-MS optimization. Chromatographic set 3 (see *section 2.4.2.2.*) employing acidic mobile phases (containing TFA) was used for LC-MS/MS experiments. The influence of the chromatographic mobile phases on the adsorption of the peptide to laboratory tube surfaces was examined by testing also the ammonia-containing solvents (chromatographic set 4) that led to similar peptide losses (data not shown). The signal intensity (relative MRM peak area – see *section 2.4.3.1.*) of hepcidin-25 was monitored during 3 to 5 consecutive injections. Each sample was freshly prepared directly before LC-MS/MS analysis, with the duration of one LC-MS/MS run of 14 min (see *section 2.4.2.2.*).

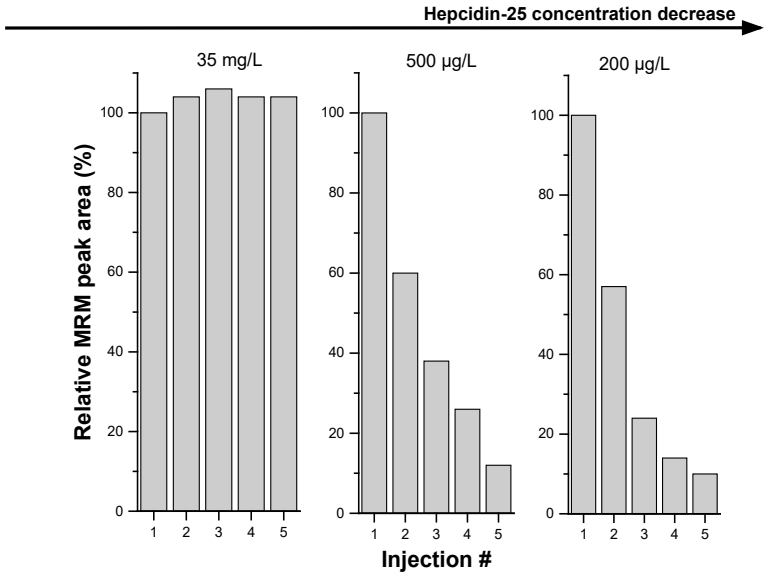

Figure 3.27: The influence of hepcidin-25 concentration on the MRM signal of hepcidin-25 of sequential injections.

A loss of signal was soon identified in the LC-MS analysis of hepcidin-25. To determine the possible causes, three parameters were tested that could influence the MRM signal alteration as

follows: The concentration of the analyte, the sample injection composition (solvent) and the vial material. Firstly, different concentrations of hepcidin-25 solutions were tested in standard HPLC glass vials. This could indicate if the signal was altered by the ionization process, independent of the concentration of the analyte. The material used was hepcidin-25 folded "in house", stored in a mixture of water/ACN/TFA 60/40/0.1 v/v/v (see *section 2.2.3.*) The results showed that the peptide losses were not significant for concentrations of 35 mg/L. However, in the case of concentrations lower than 1 mg/L, which are usually needed for preparation of hepcidin-25 standards in the desired quantification range (0.5-40 µg/L), up to 90% peptide loss has been observed (Figure 3.27). This showed that the loss of signal was not generated by ion suppression due to the additives used in the mobile phases (TFA), but was rather caused by adsorption of the peptide, for example to the walls of the glass vial. An explanation for this could be that the basic character of the 25-amino acid peptide led to electrostatic interactions with the acidic silanol groups of the glass surface.

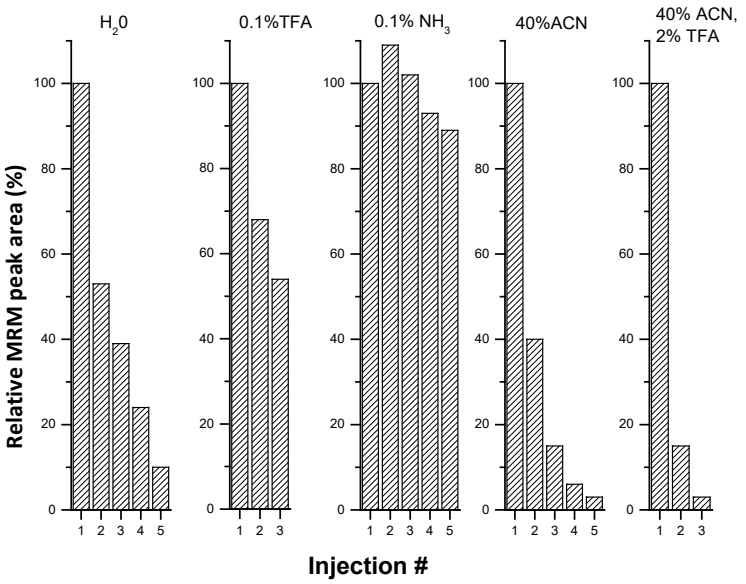

Figure 3.28: The influence of different solvents on the MRM signal of hepcidin-25 (50 µg/L). All solutions were prepared in standard autosampler glass vials.

Secondly, the preparation of hepcidin-25 solutions in different sample injection solvents using standard HPLC glass vial was investigated. Figure 3.28 shows that none of the solvents assured stable MRM signal when using autosampler glass vials. However, it could be observed that a high pH (0.1% NH_3) significantly reduced the adsorption of hepcidin to the glass surface. This phenomenon reflected the influence of pH on the electrostatic behavior of hepcidin-25. Thus, acidic solutions containing TFA cause the protonation of the peptide, and the positively charged hepcidin-25 becomes attracted by the negatively charged hydroxyl groups in the glass composition. In contrast, the basic pH of ammonia-containing solutions leads to less positive charges to interact with the silanol groups of the glass surface. Nevertheless, a mixture of wa-

ter/ACN/TFA of 60/38/2 (v/v/v) was selected as standard sample injection solution for hepcidin-25 samples not only because it facilitates a favorable MS ionization in positive mode (see *section 3.3.3.1.*), but also because it offers good compliance with the sample preparation conditions (see *section 3.3.2.3.*).

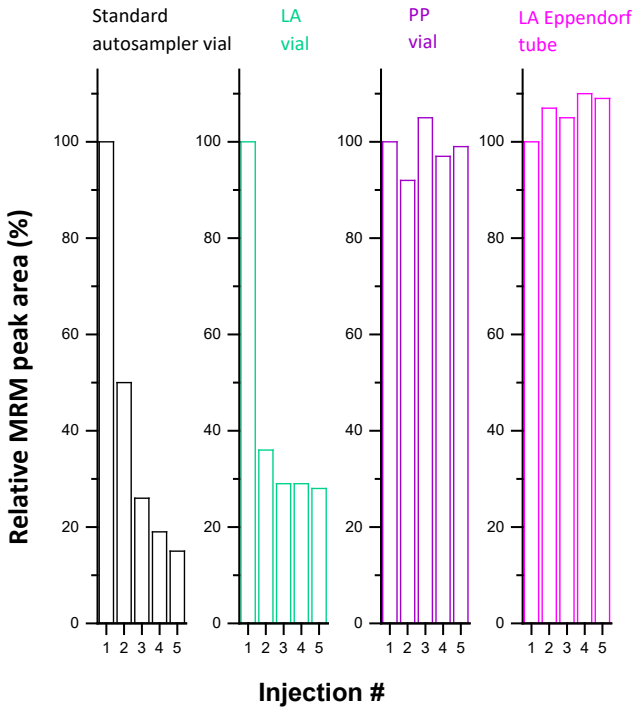

Figure 3.29: The influence of different vial materials on the MRM signal of hepcidin-25 (350 µg/L).

Lastly, the influence of vial material on hepcidin-25 concentration was investigated using the chosen sample injection solvent water/ACN/TFA 60/38/2 (v/v/v). Plastic laboratory tubes and autosampler vials of three different materials were tested for this purpose. Using standard and low adsorption commercial HPLC glass vials resulted in a respective reduction of 20% and 30% in MRM relative peak area. Plastic tubes (polypropylene vials and Eppendorf tubes) showed better suitability for storage, although the MRM signal indicated some instability (Figure 3.29). These instabilities were attributed to the non-specific interaction of the hydrophobic residues of Hep-25 with the highly hydrophobic polypropylene (PP) surface.
At this stage, it could be concluded that inert surfaces were required to reduce the interactions of hepcidin-25 with handling laboratory vessels.

3.3.2.2. The development of silanized vials

In order to reduce adsorption effects of hepcidin-25 to the autosampler vials at low concentrations (<1 mg/L), silanized vials were developed during the present work by treating standard glass HPLC vials with a 1% silane solution in toluene containing 1% of water (see *section 2.3.2.*). In this regard, it is known that glass can undergo silanization in reaction with trialcoxysilanes by hydrolysis of the silane reagent to the silanol groups of the glass followed

by surface reaction and crosslinking, leading to chemically modified glass surface. For the purpose of vial silanization, two types of silanes, namely an amino-silane (3-(2-aminoethylamino)propylmethyl-dimethoxysilane) and a fluoro-silane (1H,1H,2H,2H-perfluorooctyl-triethoxysilane) were employed (Figure 3.30).

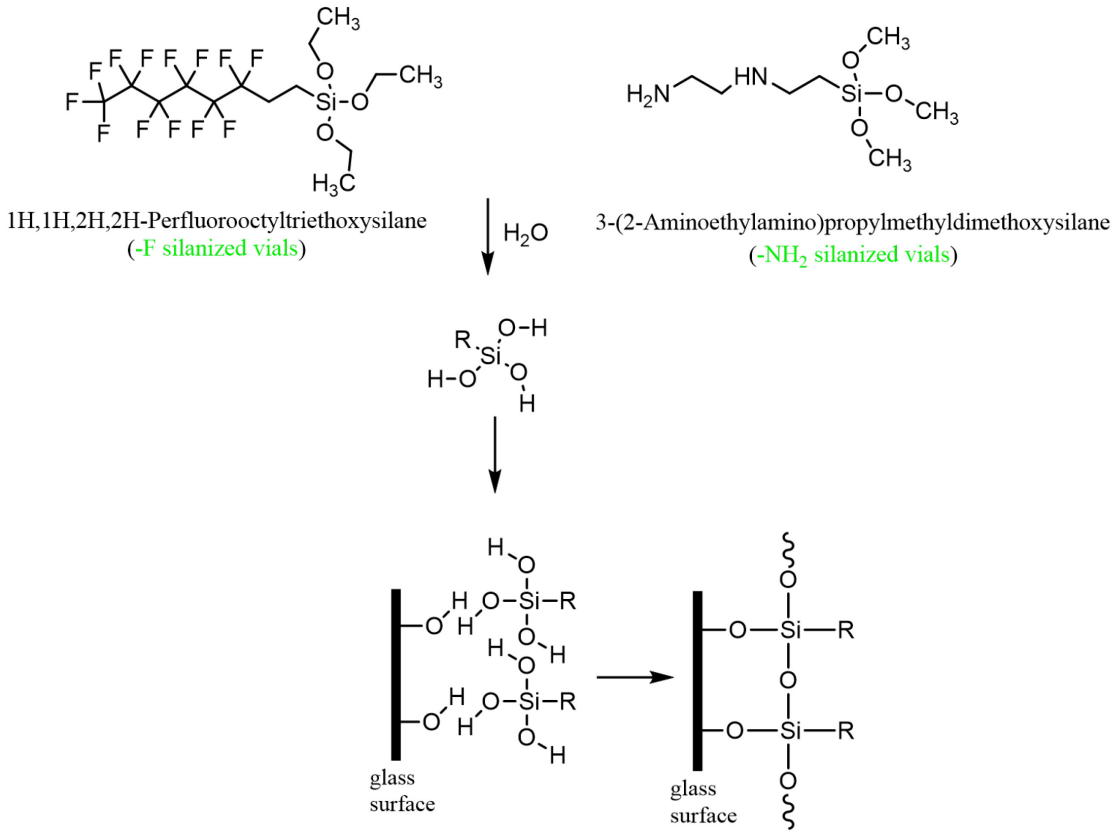

Figure 3.30: Glass silanization chemical reaction.

Adsorption effects were investigated using NH_2- and F-silanized vials, in the case of hepcidin-25 neat solution (water/ACN/TFA, 60/38/2, v/v/v) and of serum samples spiked with the peptide (Protocol I, see *section 2.3.1.2.*). All experiments were performed at peptide concentrations below 500 µg/L. Compared to commercial glass and plastic vials, the chemically modified autosampler vials showed practically no alteration in the hepcidin MRM signal, when Hep-25 solutions (350 µg/L) in water/ACN/TFA mixture 60/38/2 (v/v/v) were tested (Figure 3.31). Moreover, the inert vials were tested using several reconstitution solvents for Hep-25, with similar results (data not shown).

Additionally, when hepcidin-25 serum samples (10 µg/L) were handled in standard autosampler glass vials for LC-MS/MS analysis, the results showed MRM signal instability of hepcidin-25, probably caused by the interaction of plasma components to the walls of the vial. In this case as well, inert surfaces provided by the silanized vials permitted a stable MRM signal of the peptide for an accurate quantification (Figure 3.32).

These results enabled the conclusion that silanized glass vials represented an excellent choice of laboratory ware for hepcidin-25 handling, allowing a sensitive, precise and accurate quantification of the peptide.

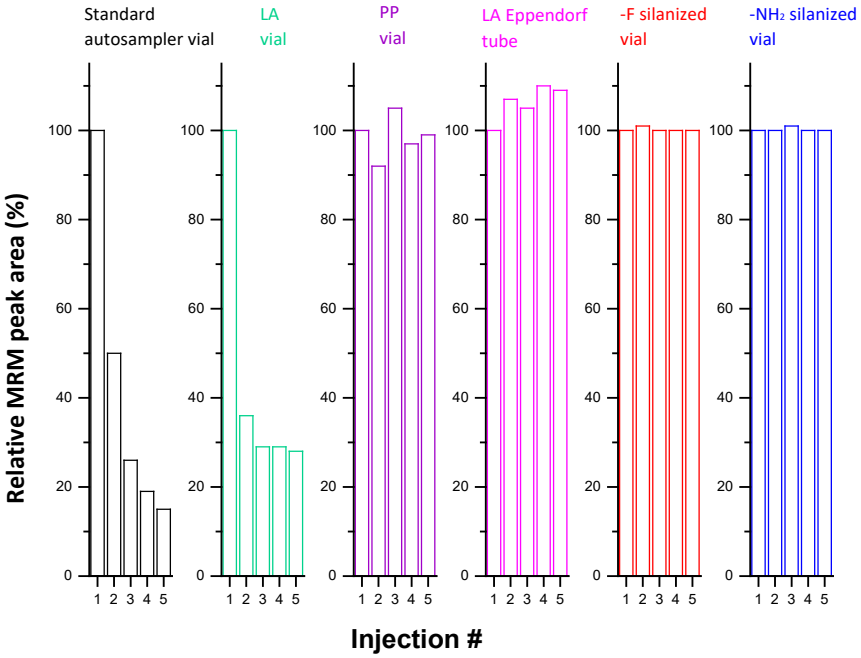

Figure 3.31: Comparison of MRM signal of hepcidin-25 solutions (350 µg/L) prepared in standard laboratory ware and in silanized glass vials (LA – low adsorption, PP – polypropylene).

It needs to be mentioned that two amino-silanes, namely 3-(2-aminoethylamino)-propylmethyl-dimethoxysilane and (3-aminopropyl)trimethoxysilane, were tested for the preparation of NH_2-silanized vials. The obtained vials showed identical behavior when used for handling Hep-25 standards (data not shown) and 3-(2-aminoethylamino)propylmethyl-dimethoxysilane was selected for further use in amino-silanization. However, differences in the performance of the amino-silanized vials compared to the fluorinated vials were found in the low concentration range (≤25 µg/L). NH_2-silanized vials proved to be the best choice when handling hepcidin standard solutions, especially at very low concentrations (10 µg/L), because the amino groups on the glass surface repel the positively charged hepcidin molecules, avoiding adsorption on the vial wall. On the other hand, F-silanized vials showed the best MRM stability in serum samples, particularly for low Hep-25 concentrations (25 µg/L) (Figure 3.33). This could be explained by taking into consideration the complex mixture of components in the matrix, which do not interact detrimentally with the surface of the non-charged inert vial. Hence, the type of silane used for surface modification should be optimized depending on the application.

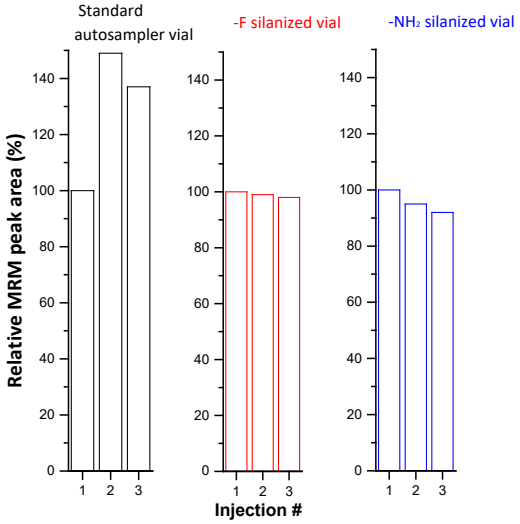

Figure 3.32: Comparison of MRM signal of serum samples spiked with hepcidin-25 (10 µg/L) prepared in standard laboratory ware and in silanized glass vials.

To conclude, the optimization of sample preparation is a key challenge in achieving a sensitive and accurate LC-MS/MS quantification of hepcidin-25. In this regard, the use of correct solvents and appropriate laboratory ware are crucial for avoiding peptide losses. Therefore, all experiments for LC-MS/MS quantification were conducted using silanized glass vials. These were also employed for the preparation of hepcidin-25 standard solutions, which was essential for the correct assignment of Hep-25 concentrations.

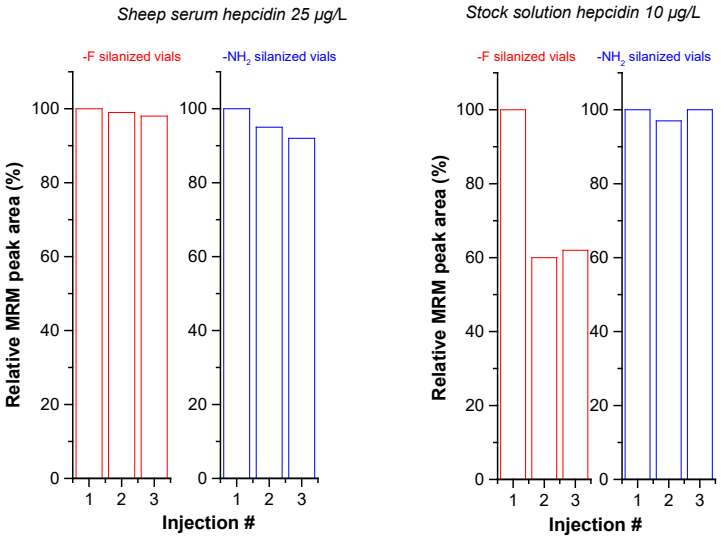

Figure 3.33: Different behavior of the F- and NH_2-silanized glass vials for neat samples and serum samples.

3.3.2.3. Serum sample preparation

For the purpose of removing or minimizing highly abundant serum proteins (such as albumin or immunoglobulins) and other serum components, which can suppress the MS ionization, different serum treatment protocols were used. Also, sample pre-concentration techniques were

employed for MS signal enhancement. The mechanisms underlying the sample preparation methods are explained below (i.-iii.).

i. Protein precipitation

The principle of precipitation relies on altering the solvation capacity of a solvent, namely by lowering the solubility of the soluble species through addition of a reagent. The amino acid content of a protein or peptide, specifically the charged residues and the hydrophilic and hydrophobic amino acids, influences its solubility. Molecules with a high content of hydrophilic amino acids possess a higher solubility in aqueous medium compared to those consisting of mainly hydrophobic residues. In addition, charged amino acids can increase the solubility of a species. When an organic solvent is added to a protein solution, solvent molecules progressively displace water molecules from the protein surface causing the decrease of its solvation layer and ultimately its aggregation due to attractive electrostatic interactions. Another technique for achieving protein aggregation is by applying isoelectric precipitation at the isoelectric point (pI) of the molecule. At a solution pH above or below its pI, the surface of a protein is predominantly negatively or positively charged respectively, and repulsion between similarly charged molecules occurs. However, at the pI value, the negative and positive charges cancel each other, and the attraction forces predominate, which consequently leads to aggregation and precipitation. Since the pI of most proteins is in the pH range of 4 to 6, organic acids such as trifluoroacetic acid or trichloroacetic acid are used in various concentrations for protein precipitation.

ii. Centrifugal filters

Membrane filters with a cut-off of 5 kDa and 30 kDa allow the removal of (most of) high-molecular weight serum compounds, while small molecules usually pass through the filter membrane and are collected for analysis.

iii. Freeze-drying (lyophilization)

Freeze-drying represents the removal of ice or other frozen solvents from a material via sublimation and the removal of bound water molecules via desorption. Usually, the serum samples are frozen by immersion in liquid nitrogen and then freeze-dried overnight.

The serum sample preparation protocols have been described in *section 2.3.1.* Protocol I was employed for LC-MS/MS analysis using TFA-based mobile phases, while protocol II was applied in the case of LC-MS/MS analysis using NH_3-based mobile phases.

Protocol I

Although both C18 [195] and weak cation exchange [197, 210] solid phase extraction (SPE) were widely suggested as sample preparation techniques for hepcidin-25 quantification, a faster and more cost-effective extraction of hepcidin from serum samples, which is compatible for routine laboratory usage, was evaluated using acetonitrile-trifluoroacetic acid-precipitated serum. Initially, protein precipitation using 40%-70% ACN was tested. However, the LC-MS/MS analysis was unsatisfactory, with no analyte detected in the obtained supernatant (data not shown). Further, isoelectric precipitation employing 2% TFA was applied additionally for serum treatment. The combination of ACN and TFA resulted in good sensitivity (LOQ of 0.1

µg/L in neat solution and 0.5 µg/L in serum), excellent recovery (99%) (see *section 3.3.4.*) and was further considered as a cheap and fast extraction procedure for the quantification of hepcidin-25 in serum samples. Hep-25 solutions containing >40% acetonitrile resulted in elution of the peptide at the injection peak (see *section 3.3.3.1.*). Therefore, 38% ACN (v/v) and 2% TFA (v/v) were applied for serum sample preparation. The supernatant obtained after protein precipitation was subsequently used for LC-MS/MS analysis, applying an injection volume (10 µL) (Figure 3.34).

Figure 3.34: Serum sample preparation - Protocol I.

Protocol II

The use of basic mobile phases (NH_3-based) was characterized by high background noise and a S/N ratio that led to a LOQ of 10 µg/L. This did not meet the requirements for hepcidin-25 quantification (dynamic range 0.5-40 µg/L) (see *section 3.3.3.2.*). For improved sensitivity, the clean-up procedure was optimized and a pre-concentration step was added. The supernatant resulted after protein precipitation employing 38% ACN and 2% TFA was further processed using 5 and 30 kDa cut-off filters (hepcidin-25, MW=2.8 kDa). Low adsorption filter membranes (Hydrosart) were used at this stage to avoid peptide losses due to the stickiness of hepcidin-25. Both filters showed improved S/N in the LC-MS/MS analysis, with the 5 kDa cut-off filters offering slightly decreased background noise as compared to the 30 kDa filters (data not shown). Therefore, these were considered for further sample preparation. The use of membranes served as an additional clean-up step to protein precipitation in the serum sample treatment. Moreover, a pre-concentration step consisting of overnight freeze-drying of the filtrate was considered (Figure 3.35). The reconstitution of the dried material led to higher concentrations of hepcidin-25 and allowed increased sensitivity for the detection of the peptide. In addition, different sample injection volumes were investigated in order to achieve an improved LOQ using Protocol II. Applying larger injection volumes represents a way to increase sensitivity. Nevertheless, too high sample injection volume affects the peak shape and resolution, and can overload the column [168]. 10, 20, 30 and 40 µL of the sample were individually injected in the LC device using the same hepcidin-25 solution (10 µg/L). An increase in the injected volume generated a relatively proportional increase in the amount of peptide. However, broadening of the MRM peak was observed when volumes > 30 µL were injected (data not shown), which adversely affects the quantification [223]. Therefore, the injection volume of 30 µL was selected in Protocol II.

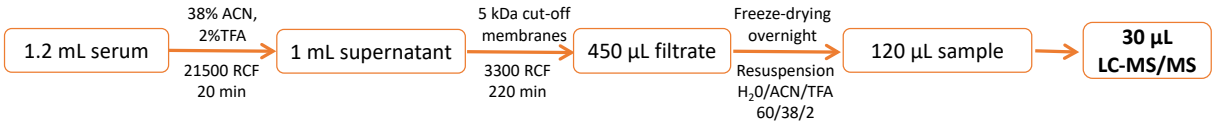

Figure 3.35: Serum sample preparation - Protocol II.

The addition of ACN (38%) and TFA (2%) applied for serum treatment was also employed for the preparation of the hepcidin-25 standards (see *section 3.3.3.1.*). This allowed the use of an "universal" solvent in the LC-MS/MS quantification of hepcidin-25. The selection of an analyte-free matrix (sheep serum) will be discussed in *section 3.3.4.1.*

3.3.2.4. Effect of storage in silanized vials on hepcidin-25

The stability of hepcidin-25 in standard solutions prepared in silanized vials was assessed over a period of 7 months to determine the suitability of the chemically-modified vials. Hep-25 standard solutions were prepared in a mixture of water/ACN/TFA 60/38/2 (v/v/v), as described in *section 2.3.1.1.*, and stored in amino-silanized vials at 4°C and -20°C respectively. LC-MS/MS analysis of the samples was performed using the TFA-based mobile phases (chromatographic set 3, see *section 2.4.2.2.*). The amount of hepcidin-25 was determined based on a 7-point calibration curve as described in *section 2.5.4.1.*

Storage at 4°C

A 400 µg/L solution of hepcidin-25 (which was generally used for the preparation of sheep standards in the low concentration range <10 µg/L) was monitored, after being store in silanized vials at 4°C for one week, one month, 4 months and 7 months (Table 3.4). Hepcidin-25 level after storage was defined as the relative ratio of the amount of peptide quantified by LC-MS/MS and the expected amount of peptide. The experiments indicated a good storage stability of the vials for at least one month. After a 7-month period however, a loss of up to 20% of hepcidin-25 was found.

Table 3.4: Relative change of hepcidin-25 stored in water/ACN/TFA 60/38/2 in silanized vials at 4°C.

Storage period	Hepcidin-25 level (%)	CV (%)
Fresh	103	12
1 week	99	5
1 month	101	9
4 months	88	5
7 months	79	6

The decrease in hepcidin-25 level after several months could be explained by taking into account its possibility of degrading into the shorter isoforms (Hep-24, -22 and -20). Studies by Laarakkers *et al.* showed that hepcidin-25 degraded into its N-truncated isoforms in human serum, when stored for more than 7 days at 4°C [93]. A faster degradation in serum could be attributed to the multitude of components present, particularly enzymes.

Storage at -20°C

Hepcidin-25 level in a 400 µg/L solution prepared in water/ACN/TFA 60/38/2 (v/v/v) was tested after 7 months of storage in NH_2-silanized vials at -20°C. No significant change was recorded in the peptide concentration. Additionally, hepcidin-25 standard solution of 1mg/L prepared in 1% acetic acid (according to the manufacturer's instructions) was tested after a 7-month period. No considerable change of the Hep-25 level was found. These results indicate that the developed amino-silanized vials represent a good choice not only for the preparation of hepcidin-25 standards (as shown in *section 3.3.2.2.*), but also for storage of the peptide calibrator solutions for a minimum of one-month duration at 4°C and a 7-month period at -20°C. This can be very useful in routine analysis in terms of saving time and costs required for the preparation of new standards per each calibration.

3.3.3. LC-MS/MS analysis

The LC-MS/MS analysis was tested by employing both acidic (TFA-based) and basic mobile phases (NH_3-based). A solution of 50 mg/L hepcidin-25 folded "in house" (water/ACN/TFA 60/40/0.1 v/v/v) was used to determine optimal MS system settings and to gain information about the ionization behavior of the peptide. Generally, the MS ionization of peptides is influenced by the amino acid content and the MS polarity applied is selected depending on the charge of the peptide in solution. In this sense, for the use of positive ion mode, the analyte is introduced in the MS device at low pH to encourage positive ion formation. Conversely, in negative ion mode, the analysis is usually carried out well above the isoelectric point of the analyte, in order to deprotonate the molecule. However, the ion behavior in the gas phase is a complex process that cannot be correlated to the pH of the sample [224]. Hence, both positive and negative modes were tested when employing acidic and basic mobile phases, respectively. The triple quadrupole was operated in full scan mode (Q1), followed by product ion mode (MS2) using CID to induce fragmentation. Multiple reaction monitoring (MRM) mode for selected transitions was applied for quantification purposes.

3.3.3.1. Acidic mobile phases - TFA-containing

As discussed in *section 1.3.2.3.*, volatile acids such as formic acid (FA), trifluoroacetic acid (TFA) or acetic acid can be used as additives in the LC-MS analysis of basic peptides. FA is usually preferred over TFA due to high suppression of the ionization efficiency of the latter one. However, TFA proved to be a preferable additive for chromatographic separation of hepcidin-25 (see *section 3.1.1.*) and a suitable precipitation reagent (see *section 3.3.2.3.*). Hence, a comparison of FA and TFA determined which additive was more suitable to be used for the quantification of hepcidin-25.

Chromatographic set 3 (see *section 2.4.2.2.*) using a C18 column was employed for the LC-MS/MS analysis. The mobile phases A (water/acetonitrile 95/5 v/v) and B (acetonitrile/water 95/5 v/v) were prepared with 0.1% TFA and FA respectively. Aiming for a fast method, suitable for routine analysis, chromatographic parameters were optimized to yield efficient separation

in the minimum time. A binary gradient with a linear increase from 5% to 95% B in 1, 2, 4, 5 and 7 minutes respectively was tested. The chromatographic conditions applying a gradient in 2 minutes showed good separation capacity and were selected further. The increase of the gradient up to 95% B was necessary due to the complex matrix used (human serum). Then, a 3-minute washing step (95% B) and a 6.5-minute re-equilibration (5% B) step were added.

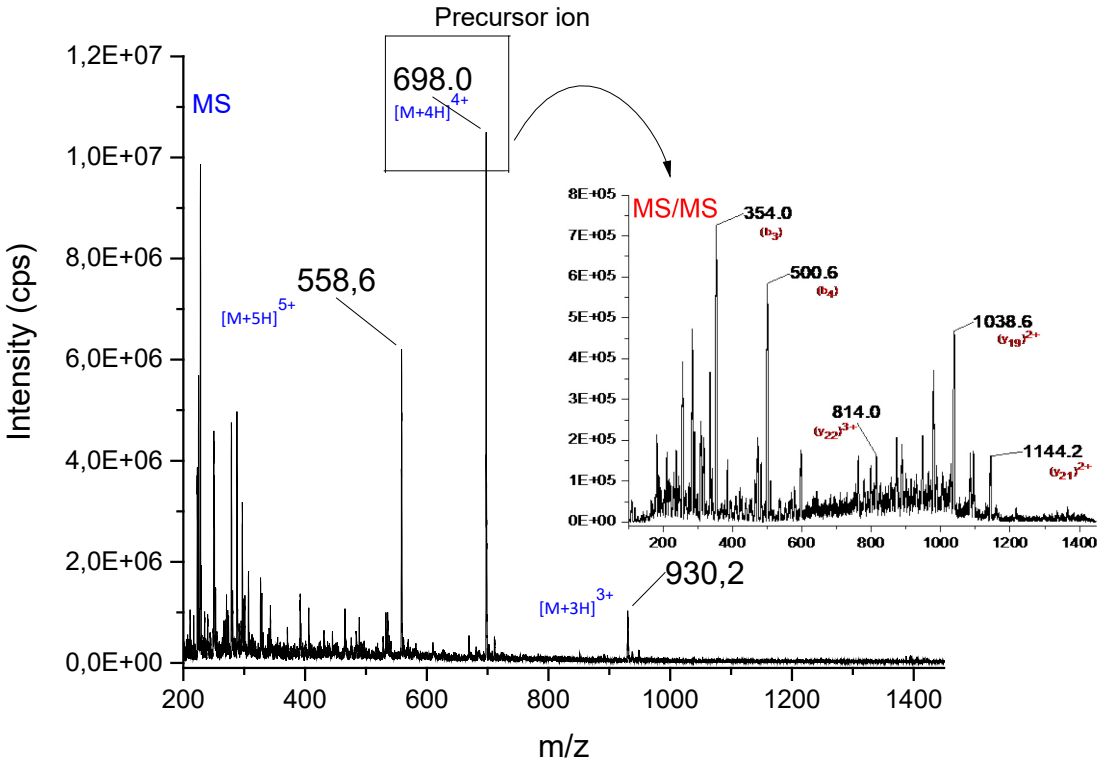

Figure 3.36: Full scan and product ion MS spectra of "light" hepcidin-25 (FA-based mobile phases).

The LC-MS/MS behavior of hepcidin-25 was highly influenced by the acidic reagent used. The chromatographic separation in the case of TFA-based mobile phases yielded a sharper peak as compared to FA-containing solvents. Moreover, the use of FA as additive in the mobile phases resulted in the quadruply charged quasi-molecular ion ($[M+4H]^{4+}$) as the most abundant precursor ion in full scan mode (Q1+), which underwent subsequent fragmentation (MS2+) (Figure 3.36). In the case of TFA-based mobile phases, $[M+3H]^{3+}$ was obtained as the most intense quasi-molecular ion that was selected further for fragmentation. The most abundant MRM transitions in the case of the two additives were further compared as follows: 698.0 → 354.0 (FA-based mobile phase) and 930.3 → 1144.7 (TFA-based mobile phases). As it can be observed in Figure 3.37, the MRM signal intensities were very similar for both TFA and FA. This means that there was no signal suppression recorded for TFA, in contrast to its general behavior reported by the mass spectrometry community (see *section 1.3.2.3.*). These findings show that the preferential use of FA as additive in LC-MS analysis is not always justified. Moreover, in

the case of hepcidin-25 quantification, the use of TFA led to a 40-fold increase in S/N as compared to FA, mostly due to low spectral background. Thus, TFA was selected as acidic additive for improved sensitivity of the LC-MS/MS analysis.

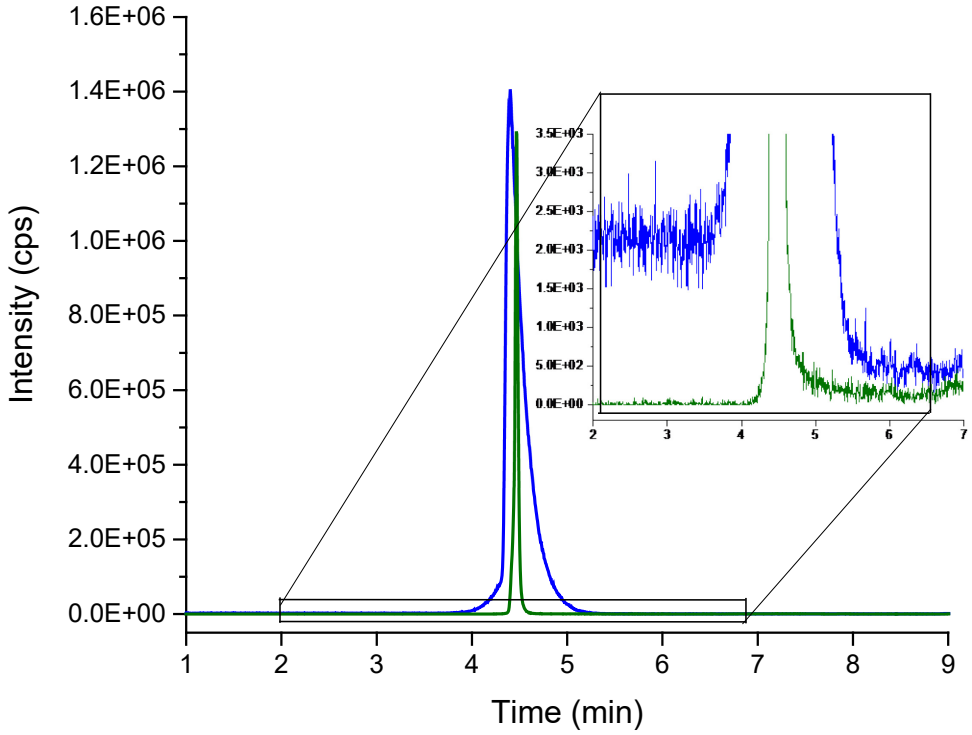

Figure 3.37: Superposed MRM chromatograms of hepcidin-25 (500 µg/L) using mobile phases containing 0.1% TFA (green trace) and 0.1% FA respectively (blue trace).

Full scan and product ion spectra of hepcidin-25 employing TFA-containing mobile phases are presented in Figure 3.38. MS analysis was carried out by operating the device in positive mode (ESI+). Negative mode ionization (ESI-) led to $[M-2H]^{2-}$ as the most abundant quasi-molecular ion, but the sensitivity was poor and no specific fragmentation could be achieved (data not shown). Mass spectrometric parameters, such as the spray ionization voltage, curtain gas and nebulizer gas, were optimized. The fragmentation pattern was highly influenced by the disulfide network, similarly as in the MS/MS analysis of hepcidin-25-copper(II) species (see *section 3.2.3.*). In this sense, the sulfur-sulfur bonds provided high stability to the biomolecule, preserved even when CID was applied. Thus, fragmentation of the triply charged quasi-molecular ion resulted in the identification, in the product ion spectra (MS2+), of b_3 (354.0), b_4 (500.4), y_{21}^{2+} (1144.7) and y_{22}^{2+} (1218.2). No full fragmentation of the precursor ion ($[M+3H]^{3+}$) was achieved, although different values for collision energy (CE) were tested for fragmentation purposes (20-60 V). However, the most abundant MRM transition (930.3 → 1144.7) that was employed as quantifier offered a very good sensitivity using a CE of 45 V. In addition to the quantifier transition, two more qualifiers were used, namely 930.3 → 1218.2 and 930.3 → 354.0.

Generally, in peptide quantification, it is recommended to use the most intense ion for quantification of the peptide, and to monitor the next two most abundant fragment ions for specificity evaluation of the assay.[201].

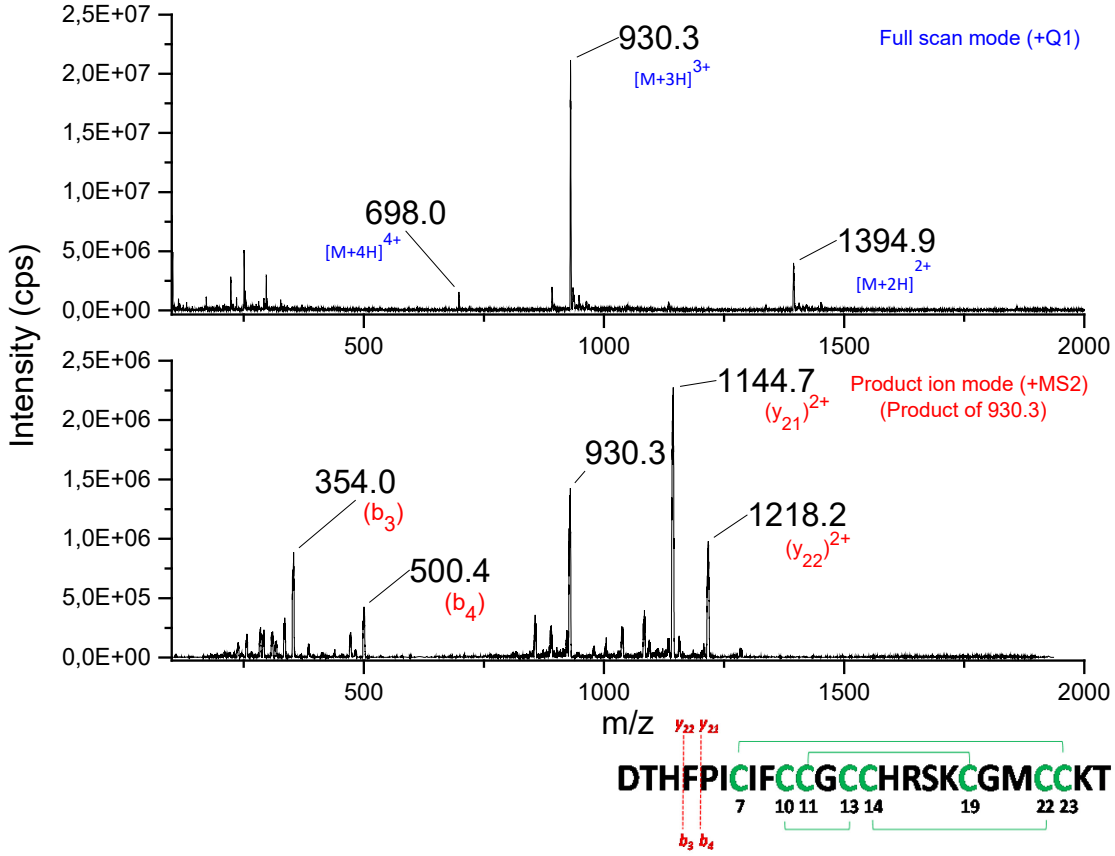

Figure 3.38: Full scan and product ion MS spectra with fragmentation pattern of "light" hepcidin-25 (TFA-containing mobile phase).

It is worth mentioning that the triple quadrupole MS was previously criticized for its poor fragmentation capacities and lack of sensitivity for hepcidin quantification [196]. However, optimized MS parameters described in this work (see *section 2.4.3.1.*) yielded a LOQ of 0.5 µg/L (see *section 3.3.4.*). It could be concluded that the triple quadrupole is a suitable mass analyzer to be employed for sensitive hepcidin quantification.

In the next step, different ratios of ACN and TFA in the solvent mixture used for the preparation of hepcidin-25 standards were tested. The results indicated that the sensitivity of the LC-MS/MS analysis was enhanced by the increase of the organic solvent ratio. This came in agreement with the results of the exploratory tests that showed a superior solubility of the peptide in the case water/ACN/TFA 60/40/0.1 (v/v/v) mixture, as presented in *section 3.1.2*. However, ACN concentrations higher than 40% resulted in the elution of the peptide in the injection peak (data not shown). Therefore, the optimum concentrations for both Hep-25 standard preparation and serum sample preparation were considered as 38% ACN and 2% TFA. Moreover, the peptide standards proved to be stable at 4°C and -20°C, when stored under these conditions (see *section 3.3.2.4.*).

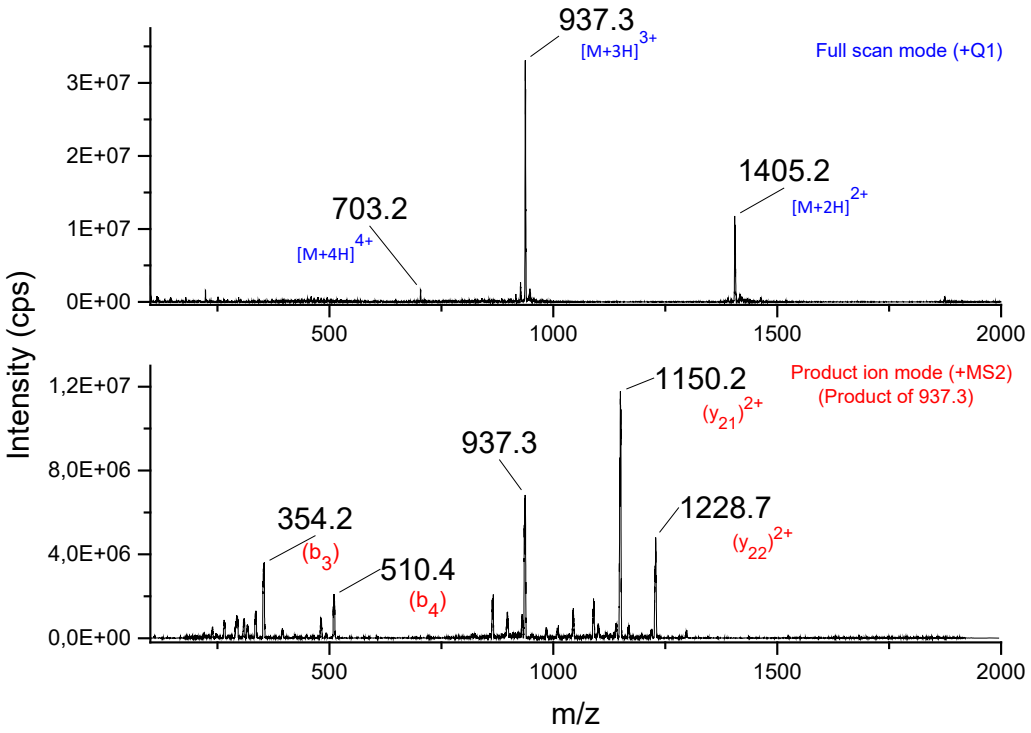

Figure 3.39: Full scan and product ion MS spectra of "heavy" hepcidin-25 (TFA-containing mobile phase).

Thereafter, the internal standard (IS), which was represented by isotope-labeled hepcidin-25 ($^{13}C_{18}$, $^{15}N_3$), was investigated using LC-MS/MS. The main advantage of using an internal standard is that, upon its addition, any possible losses due to sample handling or ionization variables are compensated, as discussed in section *1.3.2.3.* ^{13}C and ^{15}N labeled ISs generally represent a superior choice as compared to ^{2}H labeled standards. The reason behind this is that in some instances, the position of deuterium atoms can lead to hydrogen-deuterium exchange (in a mass spectrometer interface or in the gas phase), which results in alteration of the IS response and, in extreme cases, even in complete loss of deuterium atoms [168]. In addition, deuterium-based internal standards may be chromatographically separated from their "light" counterparts.

A solution of 56 mg/L isotope-labeled hepcidin-25 (also called "heavy" hepcidin-25) was considered for the LC-MS/MS analysis. The MS behavior of "heavy" Hep-25 resembled that of the "light" species. The doubly, triply and quadruply charged quasi-molecular ions were obtained in full scan mode (Q1+). The fragmentation of the most abundant ion $[M+3H]^{3+}$ resulted in the fragments b_3 (354.0), b_4 (510.4), y_{21}^{2+} (1150.2) and y_{22}^{2+} (1228.7), corresponding to the respective isotope-labeled fragments of hepcidin-25 (Figure 3.39). As in the case of "light" Hep-25, one quantifier (937.3 → 1150.2) and two qualifiers (937.3 → 1228.7 and 937.3 → 354.2) were further monitored. This LC-MS/MS method was validated using sheep serum as surrogate matrix (see *section 3.3.4.2.*), and further used for hepcidin-25 quantification in human serum (see *section 3.3.5.* and *3.4.*). Protocol I was applied for sample preparation (see *section 3.3.2.3.*).

3.3.3.2. Basic mobile phases - NH_3-containing

So far, LC-MS/MS has been applied by different research groups for the quantification of hepcidin-25, by employing reversed phase chromatography, using exclusively acidic additive-based mobile phases (see *section 1.3.2.3.*). In the present work, however, a new quantification method using ammonia-containing mobile phases (pH 11) for RP-HPLC was considered. This was based on the LC-MS/MS method developed for the chromatographic separation and MS identification of hepcidin-25-copper(II) species (see *section 3.2.2.*). The chromatographic parameters, together with the MS and MS/MS analysis of the peptide, have been presented in detail in *sections 2.2.2.* and *2.2.3.* Two HPLC columns, namely ACE Super C18 (chromatographic set 3) and ACE SuperPhenylHexyl (chromatographic set 4), were tested for hepcidin-25 analysis. Usually, phenyl-hexyl columns show remarkable selectivity for aromatic and amine compounds. C18 columns are virtually used for all HPLC applications, particularly for peptide and protein analysis. When applied to the basic mobile phases, the two columns showed analogous retention time of hepcidin-25. The phenylhexyl column was selected for further utilization. A minor retention time drift was observed, probably due to the high volatility of ammonia that could cause a slight long-term instability of the solvent mixture. Hence, a frequent exchange of the solvents was performed.

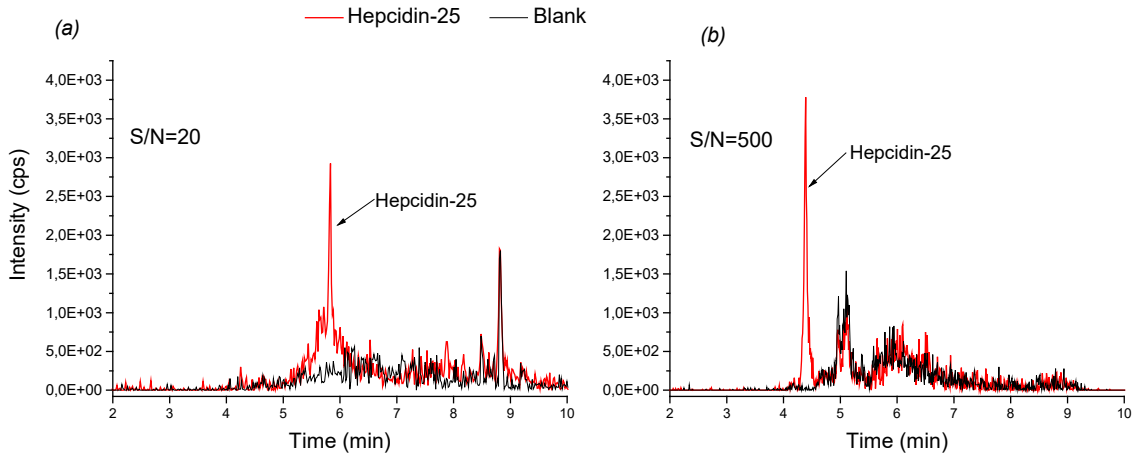

Figure 3.40: LC-MS/MS chromatogram of a sheep serum sample spiked with human hepcidin-25 (20 µg/L) using (a) basic mobile phases (0.1% NH3) and (b) acidic mobile phases (0.1% TFA).

The full scan (+Q1) MS spectra of a 50 mg/L hepcidin-25 solution revealed the triply charged quasi-molecular ion as the most abundant ion, which was further selected for fragmentation. Similarly, as in the case of acidic mobile phases (0.1% TFA), the fragments b_3, b_4, y_{21}^{2+} and y_{22}^{2+} were obtained in product ion mode (MS2+). Three transitions, namely 930.3 → 1144.7 (quantifier), 930.3 → 1218.2 (qualifier 1) and 930.3 → 354.0 (qualifier 2), were followed. One quantifier and two qualifiers were also monitored for isotope-labeled Hep-25 as follows: 937.3 → 1150.2, 937.3 → 1228.7 and 937.3 → 354.2.

The developed methodology was applied to biological matrix. The LC-MS/MS chromatogram of a sheep serum sample, spiked with hepcidin-25 solution to a concentration of 20 µg/L, showed a signal-to-noise ratio (S/N) of approximately 20, compared to the method employing TFA-containing mobile phases, which displayed a S/N of 500 for the same sample (Figure 3.40). This resulted in an estimated LOQ of 10 µg/L, which was not sufficient for the targeted dynamic range of 0.5-40 µg/L. In order to reduce the high background noise and increase the concentration of the analyte for signal enhancement, an additional clean-up step and a pre-concentration step were included in the sample preparation procedure (see Protocol II, *section 2.3.1.3.*). By such, an improved sensitivity was achieved and the method was further validated as described in *section 3.3.4.3*. Hepcidin-25 quantification in the desired concentration range was possible with both methods employing basic and acidic mobile phases (see *section 3.3.5.*).

3.3.4. Validation

3.3.4.1. Surrogate matrix

According to the CLSI guidelines, a surrogate matrix, also called "proxy" matrix, is usually required to prepare the calibration standards for endogenous analytes. The goal of surrogate matrixes is the generation of calibrators that: 1) are physiologically representative of the test matrix, thus minimizing matrix differences (matrix effects); and 2) have none or minimal concentration of analytes of interest and still retain as many physiological properties as possible [168].

In the absence of a source of human serum with no detectable hepcidin-25 levels, sera from other species were investigated as potential surrogate matrixes. To reduce the probability of interferences, the species were selected to offer a sufficient mass difference of hepcidin-25 as compared to humans (Table 3.5). Sheep and bovine serum were chosen for further investigations. However, bovine serum showed interferences with the MRM quantifier of isotope-labeled hepcidin-25 (data not shown).

Table 3.5: Hepcidin-25 mass comparison in different species.

Species	Hepcidin-25 exact mass
Human	2787.025
Rabbit	2776.116
Mouse	2752.068
Bovine	2749.062
Sheep	2703.103

In contrast, sheep serum did not interfere with the assay. Also, further investigations revealed that the produced signal suppression was lower than 35% (see *section 3.3.4.2.* and *3.3.4.3.*), which was considered acceptable. Therefore, sheep serum was further used as analyte-free surrogate matrix.

3.3.4.2. Acidic mobile phases - TFA-containing

The methods applied for evaluating the validation parameters were described in *section 2.6.* Sheep serum calibrators and quality control (QC) samples were prepared in the dynamic range of 0.5-40 µg/L using Protocol I (see *section 2.3.1.2.*).

Specificity

Two MRM transitions used as qualifiers for both "light" and "heavy" hepcidin-25 were followed for each run (sheep and human samples). The maximum permitted tolerance for relative ion intensities (% MRM qualifier/MRM quantifier) of > 20% to 50% as in the case of both "light" and "heavy" hepcidin-25 was confirmed to be within the acceptance criteria (± 25%) [83]. Also, the LC elution profiles of the qualifiers were monitored, by considering a retention time tolerance of less than 0.05 min [201].

Linearity

The calibration line (7-point calibration) based on the spiked sheep serum standards (0.5, 1, 2, 5, 10, 20, 40 µg/L) showed an r^2 value of 0.999 (Figure 3.41).

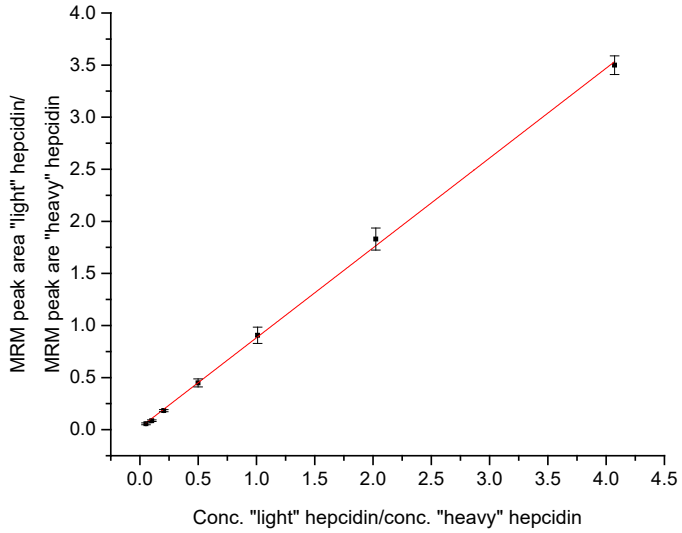

Figure 3.41: 7-point calibration curve (y=0.862x+0.020) by LC-MS/MS analysis employing acidic mobile phases (0.1% TFA) in the dynamic range 0.5-40 µg/L.

LOQ and LOD

LOQ and LOD were determined at 0.5 µg/L (Figure 3.42) and 0.15 µg/L respectively (see *section 2.6*).

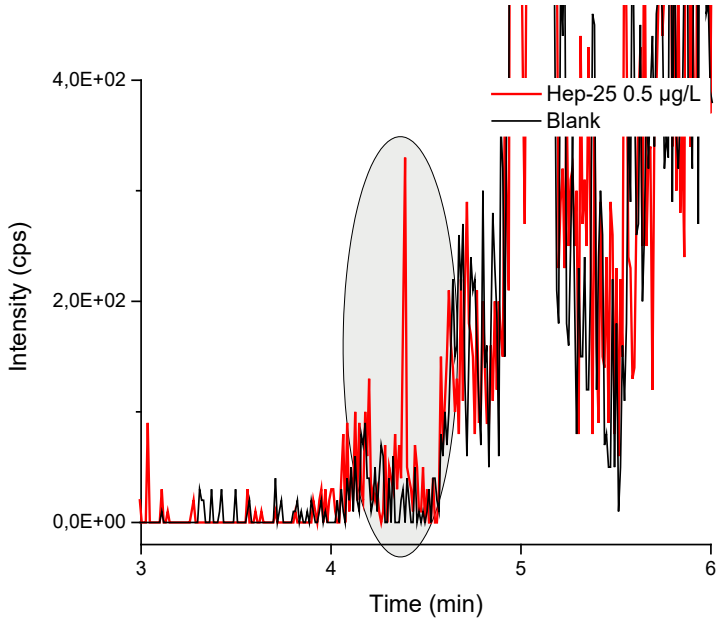

Figure 3.42: LC-MS/MS chromatograms of sheep serum samples at LOQ (S/N = 10) using TFA-containing solvents.

Precision and accuracy

Hepcidin-25^{+21} ($^{13}C_{18}{}^{15}N_3$) was used as IS to minimize errors during sample preparation and to compensate for the instrumental variations in the MS analysis.

Intra-assay precision and accuracy were assessed by analyzing triplicates of five sheep serum samples on the same day. For the evaluation of the inter-assay precision and accuracy, three quality control (QC) samples, for high (25 µg/L), medium (10 µg/L) and low (2 µg/L) level were prepared. These were then tested in five replicates on consecutive days.

Recovery and matrix effects

The recovery of hepcidin-25 after sample preparation was 99% using two QC samples (5, 10 µg/L). The evaluation of matrix effect resulted in 30% signal suppression, which was calculated by comparing hepcidin-25 peak areas in neat solution to peak areas in sheep serum.

Table 3.6: Analytical figures of merit of the LC-MS/MS quantification method of hepcidin-25 in serum using acidic mobile phases (0.1% TFA).

Hepcidin-25 conc. (µg/L)	40	20	10	2	0.5	Acceptance criteria
Intra-assay CV (%)	2.6	5.8	8.7	5.5	16.9	< 15% (LOQ < 20%)
Intra-assay accuracy (%)	-1.0	3.6	1.7	-7.6	-18.6	< ±15 %
Hepcidin-25 conc. (µg/L)	25	10	2			
Inter-assay CV (%)	7.4	7.6	7.0			< 15%
Inter-assay accuracy (%)	-4.1	-11.5	-12.4			< ±15%
Hepcidin-25 conc. (µg/L)	10	5				
Recovery (%)	99	99				> 95%
Matrix effect - signal suppression (%)	31	30				< 35%

Carryover

Carryover was observed in samples with hepcidin-25 concentration >20 µg/L. A blank injection was applied immediately after such samples to eliminate the chances of carryover to the subsequent samples. Pre-verification experiments of the unknown human samples determined at which point carryover might occur. Blank injections were introduced at this level.

3.3.4.3. Basic mobile phases - NH_3-containing

The validation procedures applied were described in *section 2.6.* The preparation of sheep serum calibrators and quality control (QC) samples was performed in the concentration range of 0.3-40 µg/L using Protocol II (see *section 2.3.1.3.*).

Specificity

Specificity was assessed as described in the previous section (see *section 3.3.4.2.*). The tolerance for relative ion intensities did not exceed ±25%.

Linearity

The standard curve was determined over the dynamic range of 0.3-40 µg/L (Figure 3.43). A linear regression was used for calibration using 8 points (0.3, 0.5, 1, 2, 5, 10, 20, 40) with an r^2 value of 0.999.

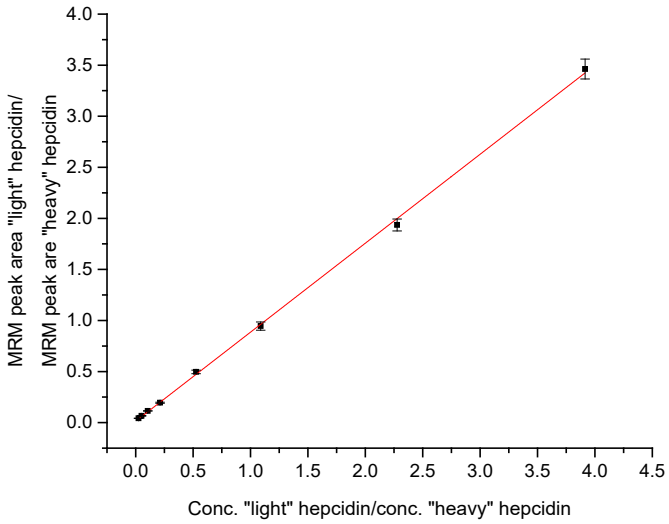

Figure 3.43: 8-point calibration curve (y=0.871x+0.014) by LC-MS/MS analysis employing basic mobile phases (0.1% NH_3) in the dynamic range 0.3-40 µg/L.

LOQ and LOD

An excellent limit of quantification (0.3 µg/L) was achieved (Figure 3.44) in case of the basic mobile phases. Moreover, a LOD value of 0.1 µg/L was obtained.

Precision and accuracy

The measurements of the spiked sheep serum samples were determined to be accurate with a relative error of <15%. Good precision was achieved with a coefficient of variation (CV) value below 15%. The recovery of hepcidin from sheep serum was 95% and matrix effects led to 30% of signal suppression.

Carryover

Carryover was assessed as described in the previous section (see *section 3.3.4.2.*).

Table 3.7 compiles the validation parameters obtained for the LC-MS/MS quantification method using basic mobile phases (0.1% NH_3) and their corresponding acceptance criteria.

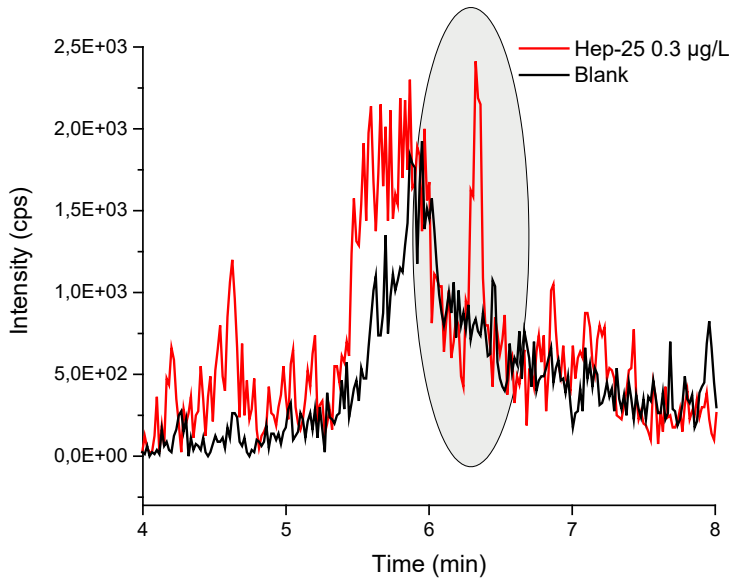

Figure 3.44: LC-MS/MS chromatograms of sheep serum samples at LOQ (S/N = 10) using basic solvents (0.1% NH_3).

In conclusion, both developed LC-MS/MS methods employing acidic and basic solvents were carefully validated and found to comply with the acceptance criteria. The method using NH_3-based mobile phases was discovered to be less robust, perhaps due to high volatility of ammonia.

Table 3.7: Analytical figures of merit of the LC-MS/MS quantification method of hepcidin-25 in serum using basic mobile phases (0.1% NH_3).

Hepcidin-25 conc. (µg/L)	40	20	10	2	0.5	Acceptance criteria
Intra-assay CV (%)	2.8	3.0	4.3	2.8	5.6	< 15% (LOQ < 20%)
Intra-assay accuracy (%)	1.1	-3.1	-1.8	-2.4	13.4	< ±15 %

Hepcidin-25 conc. (µg/L)	10	5	
Recovery (%)	95	95	> 95%
Matrix effect - signal suppression (%)	29	30	< 35%

3.3.5. Intra-laboratory comparison of two LC-MS/MS methods

Previous efforts for standardization of hepcidin-25 measurements in two international round robin exercises employed the analysis of identical human samples with different methodologies [203, 204]. The obtained discrepancies between the results were partly caused by the usage of different calibrators and the lack of a reference material, and partly because of the fact that the used immunoassays cannot distinguish the different isoforms properly, while MS-based methods selectively detect the bioactive hepcidin-25 (see *section 1.3.3.1.*). Other publications about hepcidin quantification compared newly developed MS methods to commercially available immunoassays as reference method [198, 136]. No correlation was achieved, most likely due to different analytes compared.

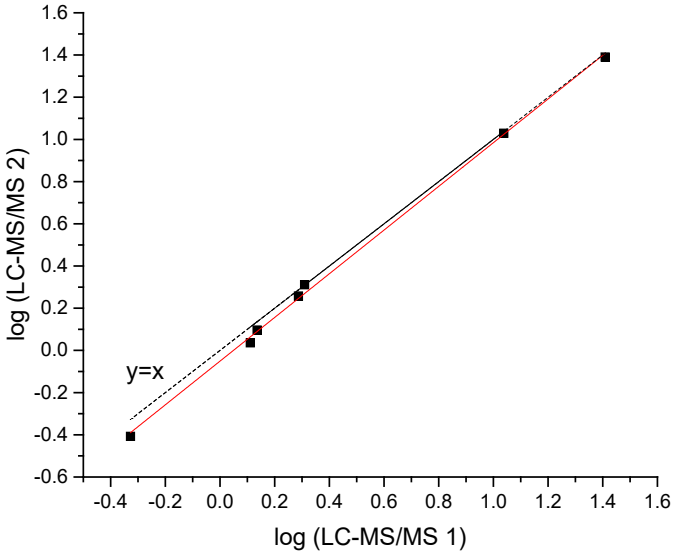

Figure 3.45: Comparison of serum hepcidin-25 levels measured by LC-MS/MS using acidic mobile phases (LC-MS/MS 1) and basic mobile phases (LC-MS/MS 2) in healthy patients, n=7 (logarithmic scale).

In the present research work, an intra-laboratory comparison of two independent MS-based assays for the quantification of hepcidin-25 was performed using the same calibrators to assess the accuracy of these methods. The two LC-MS/MS methodologies described in the previous sections, namely the method employing acidic (see *section 3.3.3.1.*) and basic mobile phases (see *section 3.3.3.2.*), were applied to nine human samples for the assessment of hepcidin-25. The amount of peptide in native samples was determined using a 7-point and 8-point calibration curve respectively, as presented in *section 2.5.4.1.* The serum levels of hepcidin-25 varied in a broad range. Therefore, a logarithmic scale seemed suitable for the comparison of the methods. A significant positive correlation (r^2=0.999) between the two LC-MS/MS methods was found (Figure 3.45) and the Bland-Altman plot confirmed the commutability of these assays (Figure 3.46). It should be noted here that the samples with concentrations lower than LOQ (n=2) were not considered for the correlation.

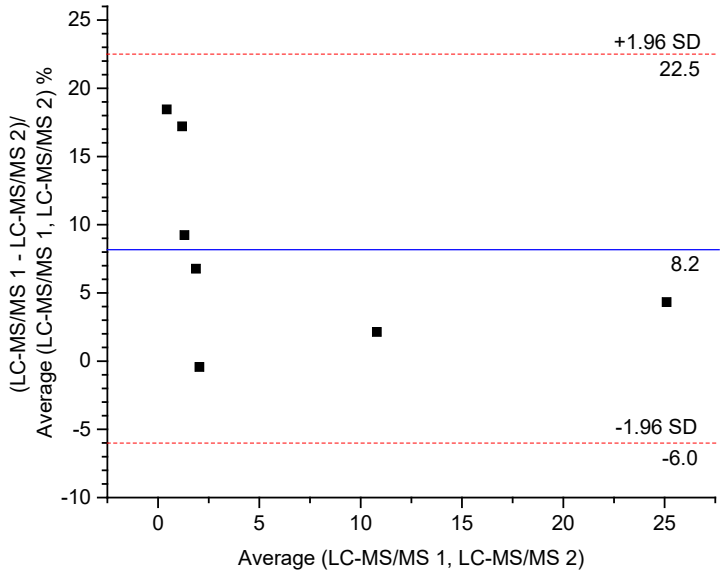

Figure 3.46: Comparison of serum hepcidin-25 levels measured by LC-MS/MS using acidic mobile phases (LC-MS/MS 1) and basic mobile phases (LC-MS/MS 2) in healthy patients, n=7 (Bland Altman plot).

These results suggested that both methods are suitable for hepcidin-2 quantification, particularly when a reference material is available.

3.4. Reference method candidate for clinical assessment of hepcidin-25 in human serum

3.4.1. Recommended LC-MS/MS method for hepcidin-25 quantification in routine laboratories

Two sample preparation strategies and two chromatographic separation conditions have been investigated with the aim of developing a LC-MS/MS method that could serve as a reference method for the sensitive and reliable quantification of hepcidin-25, and could be implemented in routine laboratories. In order to be able to recommend a methodology in this sense, several clinically-relevant criteria were evaluated, including the time of the analysis, the costs involved and the sample volume required. These aspects were considered in addition to the analytical figures of merit that have been discussed previously (see *section 3.3.4.*).

Due to short sample preparation time, small sample volume necessary for the analysis, relatively reduced hepcidin-25 extraction costs and superior robustness (see *section 3.3.4.3.*), the LC-MS/MS method employing TFA-containing mobile phases is proposed as the methodology for routine analysis in the clinical field. An overview of the method parameters and a critical evaluation of the measurement uncertainty will be presented further.

Table 3.8: Correlation between quantification methods and selected criteria.

Criteria relevant for clinical use	LC-MS/MS using TFA-based mobile phases	LC-MS/MS using NH_3-based mobile phases
Sample preparation time	+++* (10 min centrifugation)	+* (20+220 min centrifugation, overnight freeze-drying)
Sample volume required	+++* (300 µL)	+* (1.2 mL)
Sample preparation costs	+++* (ACN, TFA)	++* (ACN, TFA, Hydrosart membrane filters, liquid nitrogen)

* *Very good +++, good ++, fair +*

3.4.1.1. Scheme of the reference method candidate for hepcidin-25 quantification

Figure 3.47 represents a scheme comprising the three essential steps of the developed LC-MS/MS reference method candidate. First, amino- or fluoro-silanized glass vials were employed to avoid hepcidin-25 losses due to its sticky character. Cost-effective extraction of the peptide from serum samples was achieved applying acetonitrile (38%)-trifluoroacetic acid (2%)-precipitated serum. Then, reversed phase chromatographic separation, by using a highly inert C18 column, was obtained. Finally, sensitive and precise MS/MS detection was achieved by employing a triple quadrupole mass spectrometer being operated in MRM mode.

3.4.1.2. Uncertainty budget of the reference method candidate

The measurement uncertainty as defined by CLSI is a non-negative parameter, which characterizes the dispersion of the quantity values being attributed to the analyte, based on the information used. This parameter may be, for example, a standard deviation called standard measurement uncertainty or the half-width of an interval, having a stated coverage probability [168]. To determine the uncertainty of the measurement, one must determine the sources of variability (random error) and bias (systematic error).

In the case of the developed LC-MS/MS methodology for the quantification of hepcidin-25, the main elements which influence the precision and accuracy of the method are the sample preparation, the ionization process and the purity of the standards.

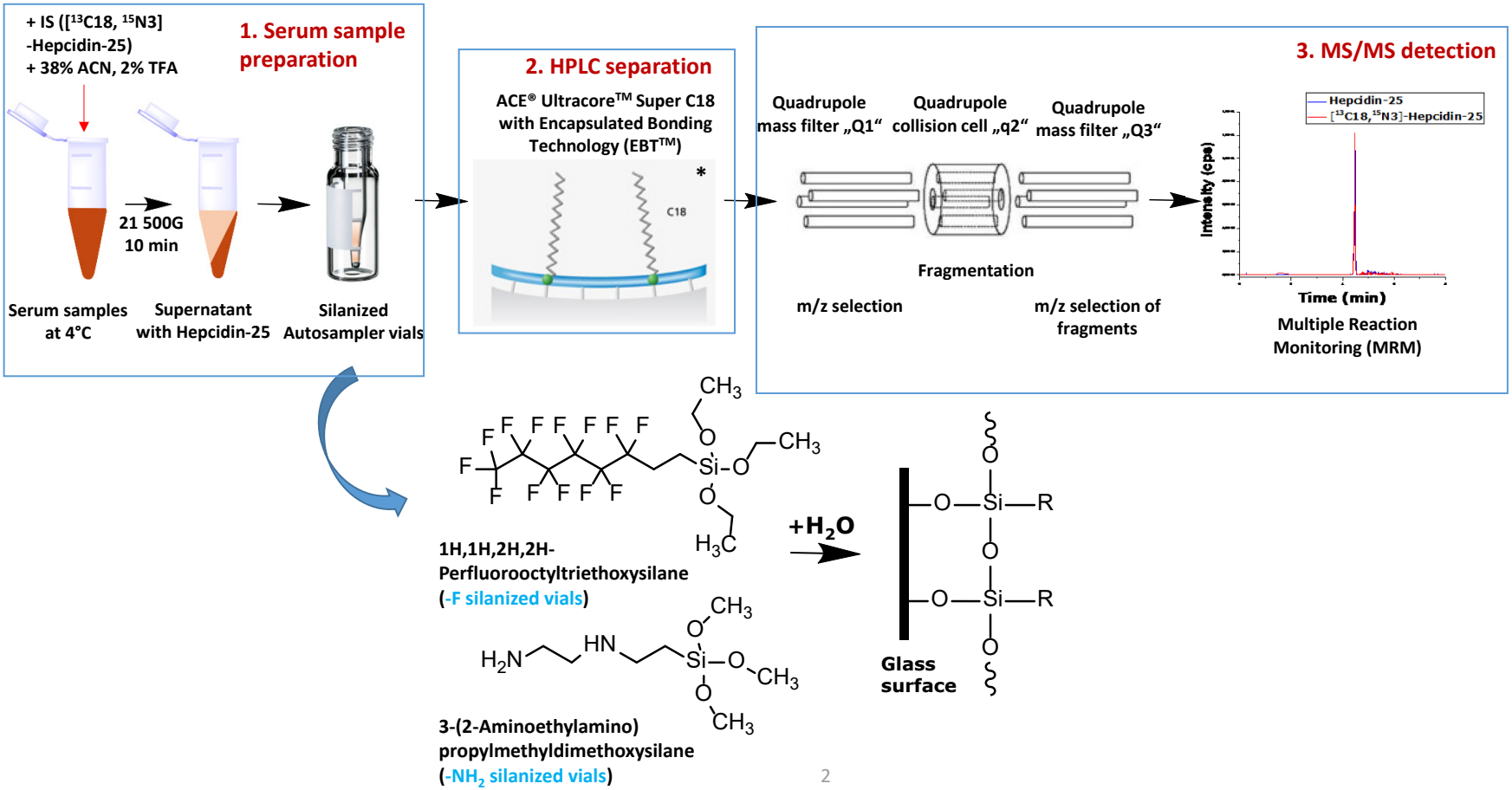

*Figure 3.47: Work flow for the reference method candidate for hepcidin-25 quantification in human serum (*Reproduced with permission of Advanced Chromatography Technologies Ltd, UK).*

Random error - precision

As mentioned before, "heavy" hepcidin-25 is used as IS to correct the loss of analyte during serum treatment (protein precipitation) and the matrix effects during analysis. Thus, the precision of the measurement is mainly influenced by the reproducibility of the MS signal, given by the standard deviation of the LC-MS/MS analysis. By measuring triplicates, this was determined to be <15% (<20% at 0.5 µg/L), as described in *section 3.3.4.2*.

Systematic error - accuracy

The accuracy of the method is significantly influenced by the purity and the preparation of the standards. In this regard, the type of calibration used determines if the "light" or "heavy" hepcidin-25 contributes to the accuracy. Generally, two approaches are popular for peptide calibration, namely single-point calibration and the use of standard curve. In the single-point calibration, a known quantity of the IS (isotope-labeled peptide) is added to a sample and then the signal of the target analyte is measured relative to the internal standard, by taking into consideration the so-called response factor (see *section 1.3.2.3.*). This approach demands high chemical purity and isotope enrichment of the IS. By such, the internal standard influences the accuracy of the measurements [194]. However, several limitations in the use of a single-point calibration, such as poor day-to-day reproducibility and the inability to confirm that the measurements are within the linear range, restrict its application in routine analysis [194, 201]. Therefore, the standard curve approach was selected in this study. The CLSI guidelines indicate that calibration curves which represent ranges of up to three orders of magnitude should incorporate 6 to 8 standards [168]. Thus, a 7-point calibration curve was considered for the quantification of unknown samples in the dynamic range of 0.5-40 µg/L. In this respect, the MRM intensity of the analyte ("light" hepcidin-25), normalized to the internal standard ("heavy" hepcidin-25), was plotted against the corresponding concentration ratio. A constant amount of "heavy" hepcidin-25 (10 µg/L) was added to several "light" hepcidin-25 standards of known quantity that spanned the selected dynamic range. This approach implied that the "heavy" hepcidin-25 improved the quantitative precision, while the accuracy of the results relied on the purity of "light" hepcidin-25, in contrast to the single-point calibration. In this regard, the standard solutions of hepcidin-25 were prepared gravimetrically as described in *section 2.3.1.1.* to lower the error compared to pipetting [168].

Uncertainty budget

To conclude, the elements contributing to the uncertainty budget are the standard deviation of the LC-MS instrumentation (up to 15%), the purity of hepcidin-25 as stated by the manufacturer (> 95%, HPLC analysis) and the uncertainty of the balance (approximately 0.01-0.5% in the range 20-1000 mg). Being majorly influenced by the standard deviation values, the measurement uncertainty can also be referred to as the standard deviation (coefficient of variation) of the LC-MS analysis.

3.4.2. Hepcidin-25 levels in healthy individuals

The chosen LC-MS/MS methodology employing acidic mobile phases (0.1% TFA) was applied to determine variations in hepcidin-25 levels in healthy donors. Human sera from nine individual subjects, in the age ranging from 24 to 54, were analyzed. The amount of Hep-25 in the unknown samples was determined by using isotope-labeled hepcidin-25 for internal calibration and a 7-point calibration curve in the dynamic range of 0.5-40 µg/L for external calibration (see *section 2.4.5.1.*).

The concentration of hepcidin-25 in the clinical samples ranged from <0.5 µg/L to 25.6 µg/L. The mean (arithmetic mean) and median values were calculated by considering the two samples < LOQ with an assigned value of 0.25 µg/L (LOQ/2). A median of 1.9 µg/L and a mean value of 6.2 µg/L were obtained. The standard deviation for the mean value was 9.3.

Table 3.9: Physiological level of hepcidin-25.

Affiliation	Quantification method	Patient no	Mean (µg/L)	Median (µg/L)	Concentration range (µg/L)	Ref.
Eli Lilly and Company	LC-MS/MS (Triple Quadrupole)	10	9	3.6	<1.0 - 45.6	[195]
	Sandwich ELISA	100	N/A	1.2	<0.02 - 25 (44% < 0.9 µg/L)	[144]
Present work	LC-MS/MS (Triple Quadrupole)	9	6.2	1.9	<0.5 – 25.6 (33% < 0.9 µg/L)	[225]

The physiological level of hepcidin-25 in humans seemed to vary widely, as has been extensively reported in literature [144, 136] (see *section 1.3.3.3.*). The results fitted the values established previously (physiological median level of 1-5 µg/L and mean level of 5-15 µg/L) (see *section 1.3.3.3.*). Moreover, the obtained values were found to be directly in line with the results reported by the research group of Eli Lilly and Company (Table 3.9).

The developed LC-MS/MS method represents a suitable reference method candidate for accurate hepcidin-25 quantification in human serum, particularly when a certified reference material (CRM) will be available. In this regard, the calibration of the method by applying a secondary reference material (sRM), which was developed in the frame of the ongoing hepcidin-25 harmonization studies (see *section 1.3.3.1.*), will be described in the following section.

3.4.3. Participation in the international Hepcidin Harmonization Study (HHS2017)

Hepcidin-25 harmonization efforts, carried out by Radboud University Nijmegen and hepcidinanalysis.com, identified a commutable secondary reference material (sRM), which

consisted of native lyophilized serum with cryolyoprotectant, to be used for hepcidin-25 quantification [132]. In the next step, the Hepcidin Harmonization Study 2016 (HHS2016) aimed at the validation of commutability of this sRM and its functionality to increase the degree of equivalence between measurement procedures for hepcidin-25 quantification. The conclusion of HHS2016 was that harmonization is indeed possible with the use of the newly identified sRM. The commutability of the reference material was approved. Also, the validity of the calibrator for harmonization purposes was confirmed through mathematical simulations [214].

Finally, Radboud University Nijmegen and hepcidinanalysis.com organized a round robin study in June 2017 (Hepcidin Harmonization Study HHS2017) to establish whether harmonization could be achieved. The secondary reference material consisting of two levels (middle and low) and native samples of three levels (high, medium, and low) were considered for the study. The participants were asked to measure the values of the native samples by applying the sRM for calibration. In order to lower the bias, the organizer's request was to have the samples measured in triplicate within one run. Statistical methods were applied at Radboud University Nijmegen to determine the proximity of data of the sample values, and to find out whether harmonization has been achieved. The LC-MS/MS method employing TFA-containing mobile phases developed within the present work was one of the ten methodologies within the HHS2017. In the next sections, investigations on the calibrator set and on the native samples will be described.

3.4.3.1. Secondary reference material determination

The two constituents of the calibrator set (level low and medium) were quantified using the developed LC-MS/MS methodology based on a 7-point calibration curve (see *section 2.5.4.2.*) and were consequently compared to the assigned value (Table 3.10). This assigned value was selected by a consensus approach, based on the guidelines of the International Consortium for Harmonization of Clinical Laboratory Results [214, 226], as a mean value of 9 validated methods worldwide, namely 5 MS-based methods and 4 immunoassays [214].

Table 3.10: Calibrator set values [214].

Calibrator level	Mean value (SD) of 9 validated methods (II) **[µg/L]** / *nM*	Obtained value (SD) by BAM Berlin (III) **[µg/L]** / *nM*
1 (low)	**6.65 (3.41)** *2.38 (1.22)*	**3.48 (0.22)** *1.25 (0.08)*
2 (medium)	**19.64 (8.80)** *7.03 (3.15)*	**9.90 (0.17)** *3.55 (0.06)*

Although the two compared values showed differences, a constant comparative factor of 0.5 was obtained, when expressing the value recorded in this study (BAM Berlin) relative to the consensus value. This shows that the deviations between aforementioned values were

actually most likely caused by the differences in absolute values of the calibrators, rather than the methodology used.

3.4.3.2. Native samples quantification

In the next step, three unknown human samples were determined by LC-MS/MS using two quantification approaches (without and with the use of sRM), applied in different analytical runs as described in *section 2.5.4.2*. (Table 3.11).

Table 3.11: Hepcidin-25 quantification approaches.

	Quantification without sRM	**Quantification with sRM**
Internal calibration	Commercial "heavy" hepcidin (Peptide Institute)	
External calibration	Commercial "light" hepcidin (Bachem) 7-point calibration curve	Calibrator set (hepcidinanalysis.com) 2-point calibration curve

As expected, the ratio between the results of the above-mentioned quantification approaches stayed the same as the comparative factor of 0.5 obtained from the calibrator set determination. This confirmed that the discrepancies between the results were basically because of different calibrators used. At this point, it is difficult to say which material would be more suitable for an accurate assignment of hepcidin-25 reference levels, the commercially available standard with a reported HPLC purity >95% (Bachem, Bubendorf, Switzerland) or the calibrator set with a consensus value obtained as a mean of 5 MS-based assays and 4 immunoassays. Nevertheless, a primary reference material, also known as certified reference material (CRM) is required for the assignment of absolute hepcidin-25 values, which would ensure metrological traceability to the International System of Units (SI).

The performance of the LC-MS/MS method was compared to the other participating methods. Figure 3.48 shows the degree of equivalence between the results of the ten selected methods worldwide, expressed as the total allowable error (TEa) based on the recommendations of the International Consortium for Harmonization of Clinical Laboratory Results [226]. These results represented the quantification values obtained for the three unknown samples by applying each method. Because of the absence of a true value, the x-axis represents the mean results of the samples for all methods. The y-axis shows the bias, that is to say the difference between results of individual samples of each method and the mean of all methods. The lines represent limits for optimum, desirable and minimum TEa. Under these conditions, the LC-MS/MS method developed within this study stayed within the optimal TEa.

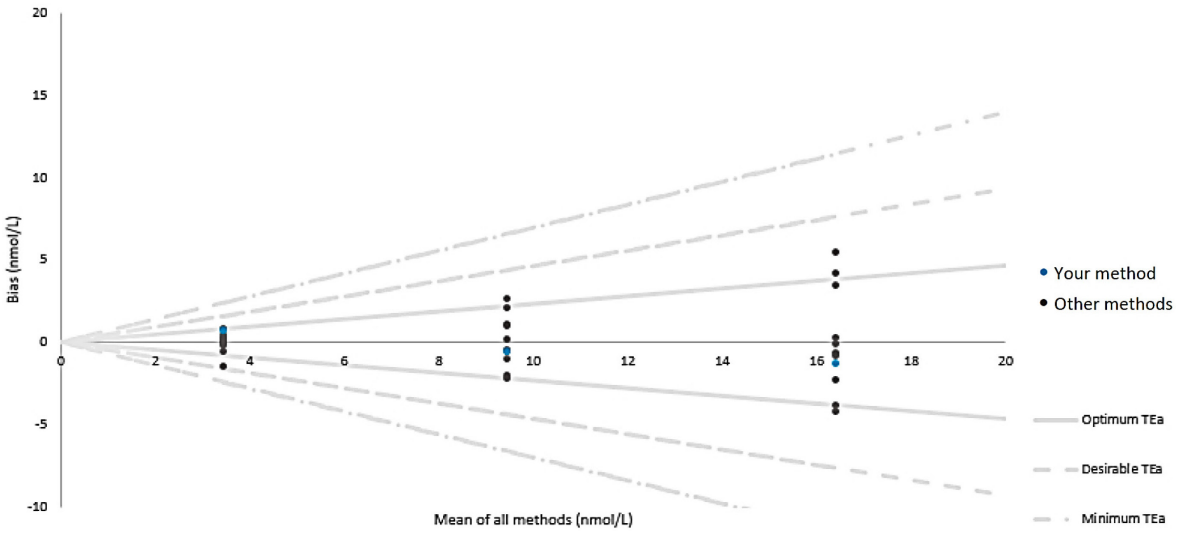

Figure 3.48: Degree of equivalence between measurement results, expressed as total allowable error (TEa), obtained after calibration with sRM. Blue dot – LC-MS/MS method by BAM Berlin (Figure provided by Dorine Swinkels, Radboud University Nijmegen).

These findings support the notion that the developed reference method candidate, compared to the other existing quantification methodologies, represents an excellent choice for the quantification of hepcidin-25 in native samples. In this context, it is worth mentioning that the method developed in this research work (at BAM Berlin) was the only LC-MS/MS method participating in HHS2017 from Germany.

Conclusions and Outlook

The main scope of this study was to develop and validate a reliable tool for the quantification of hepcidin-25 in human serum. For this purpose, the characterization of the analyte in terms of its structure, solubility and copper-binding properties was achieved and different quantification strategies were tested.

As a starting point, a methodology was developed for the "in house" folding of linear hepcidin-25, resulting in one sole folded product, as determined by HPLC. The comparison of its retention time and its MS isotope pattern to a commercial hepcidin-25 standard confirmed the identity of the folded 25-amino acid peptide with a reported disulfide connectivity of Cys7-Cys23 (1-8), Cys10-Cys13 (2-4), Cys11-Cys19 (3-6) and Cys14-Cys22 (5-7). The folding procedure showed good yields (10-50%) and was further applied to produce multi-milligram amount of the peptide. In future, ion mobility mass spectrometry IM-MS could be employed as a complementary technique able to separate isomers and for further investigation of partial and total oxidation of the cysteine residues. Also, the disulfide connectivity should be attributed to the folded products (in different stages of the folding process), using for example partial reductive alkylation.

In addition, the solubility of the peptide in different solvents and at different pH values was tested using HPLC. The peptide was found to be most soluble in acidic solutions (1% acetic acid or 0.1% TFA) using polar solvents (40% ACN). This was later confirmed by LC-MS/MS analysis. Also, the addition of methanol (20-50%) improved the solubility of hepcidin-25, particularly in the pH range of 7.4 to 8.5. In this regard, the synthesis of labeled hepcidin-25 with a fluorescent dye was achieved at a pH of 8.5 by using 50% methanol (v/v).

Moreover, the first chromatographic separation of hepcidin-25 and its complexes with copper(II) was achieved by employing ammonia-containing mobile phases (pH 11) and phenyl-hexyl HPLC columns with extended pH stability. LC-MS/MS investigations of hepcidin-25 complexes with copper(II) ions using the triple quadrupole revealed two species at a pH of 11.2, namely hepcidin-25/Cu^{2+} and hepcidin-25/2Cu^{2+}. HR-MS (FTICR-MS) confirmed the isotope patterns of the two compounds and established that the first copper ion binds the peptide with the loss of two H^+, while the second copper interacts with hepcidin-25 without any deprotonation. Furthermore, at pH 7.4, the mono-copper(II) species is preferentially formed. Additional NMR structural analysis permitted the development of the first 3D-model of the Cu^{2+}-bound hepcidin-25. The results of these investigations (LC-MS/MS, FTICR-MS and NMR) clearly support the hypothesis that hepcidin-25/Cu^{2+} represents the native form of the iron regulator in the human body.

However, sample preparation techniques that preserve the metal complex (pH>5) need to be further developed in future for the LC-MS identification/quantification of the copper peptide in biological samples. Additionally, the bioactivity of hepcidin-25/Cu^{2+} should be compared to hepcidin-25, (by monitoring the internalization of ferroportin) to determine, if a copper(II)-assisted modulation of biological activity occurs in the case of hepcidin-25.

Lastly, the steps required for the LC-MS/MS quantification of hepcidin-25 in human serum, namely sample preparation, chromatographic separation and MS/MS detection, were investigated and optimized. In this regard, it was shown that special laboratory ware is needed for storage and handling of the hepcidin-25 stock solutions and for the assessment of the peptide in human serum, because the charge of the peptide and its amphipathic character make it readily stick to plastic or glass surfaces. Silanized vials were developed, which provided inert surfaces able to reduce the adsorption of the peptide to the walls of the autosampler vials (at concentrations below 1 mg/L). It was found that using glass-based equipment led to significant peptide losses. Applying plastic-based laboratory ware instead caused lower adsorption effects, however, some instability of the signal was recorded. On the contrary, amino- and fluoro-silanized vials showed practically no MS (MRM) signal instability and could be used for precise, accurate and sensitive peptide quantification. Amino-silanized vials were employed throughout this study for preparation and storage of hepcidin-25 standard solutions and fluoro-silanized vials were utilized for LC-MS/MS analysis of serum samples.

Two isotope dilution (ID-)LC-MS/MS methodologies were developed and validated for the quantification of hepcidin-25 in human serum by applying isotope labeled hepcidin-25 ($^{13}C_{18}$, $^{15}N_3$) as an internal standard. The triple quadrupole mass spectrometer operated in MRM mode was successfully employed for this purpose. The first method used acidic mobile phases (0.1% TFA) and a fast and cost-effective peptide extraction based on acetonitrile-TFA precipitated serum (38% and 2% respectively). The second method employed basic mobile phases (0.1% NH_3) at a pH of 11 and a more complex sample preparation protocol, comprising protein precipitation and ultrafiltration as clean-up steps, followed by freeze-drying for pre-concentration purposes. The two quantification methods were validated over the dynamic range of 0.5-40 µg/L (TFA-based mobile phases) and 0.3-40 µg/L (ammonia-containing mobile phases) in accordance with the guidelines of the Clinical and Laboratory Standards Institute (CLSI). A Bland-Altman plot validated the commutability of the two assays, when these were applied to human serum.

Due to quicker sample preparation and superior robustness, the LC-MS/MS method using acidic mobile phases was chosen as a reference method candidate for the quantification of hepcidin-25 in serum samples. The method presented very good analytical figures of merit in terms of sensitivity (LOQ 0.5 µg/L) and reproducibility (CV <15%), and was shown to

be suitable for routine analysis. The method was applied to assess the variability of hepcidin-25 in healthy subjects. A mean physiological level of approximately 6 µg/L (concentration range <0.5-25 µg/L) and a median value of 2 µg/L were determined. These results were found comparable to previously reported values obtained by Eli Lilly and Company by employing two different analytical techniques (a sandwich ELISA and a LC-MS/MS method using a triple quadrupole system). Additionally, the recommended reference method candidate stayed within the optimum total allowable error as defined by the International Consortium for Harmonization of Clinical Laboratory Results in an international harmonization study (HHS2017) organized by Radboud University Nijmegen and hepcidinanalysis.com, which assessed the degree of equivalence between the results of ten validated quantification methods by using a secondary reference material as calibrator set. Five immunoassays and five MS-based techniques were compared in this study.

In future, the assignment of the absolute levels of hepcidin-25 in healthy individuals using a primary reference material (or certified reference material (CRM)) is desired to reach hepcidin-25 standardization. A candidate, in this vein, is under development at LNR (Laboratoire National de métrologie et d'Essais), Paris, France. Physiological and pathological reference ranges of hepcidin-25 should be assessed by a reference method (calibrated using a CRM) in order to define clinical decision limits.

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Selbstständigkeitserklärung

„Ich erkläre, dass ich die Dissertation selbständig und nur unter Verwendung der von mir gemäß § 7 Abs. 3 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät, veröffentlicht im Amtlichen Mitteilungsblatt der Humboldt-Universität zu Berlin Nr. 126/2014 am 18.11.2014 angegebenen Hilfsmittel.“
angefertigt habe.“

Berlin, den 23.05.2018
Ioana-Monica Abbas